# THE EFFECTS OF TEMPERATURE ON DEVELOPMENT AND GROWTH OF MUSCLE IN THE TROUT (SALMO TRUTTA (L.))

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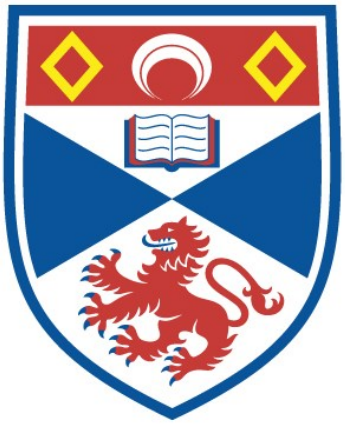
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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# THE EFFECTS OF TEMPERATURE ON DEVELOPMENT AND GROWTH OF MUSCLE IN THE TROUT (*SALMO TRUTTA* L.)

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# Abstract

Trout (*Salmo trutta* L.) were reared in a hatchery environment under a variety of temperature regimes, and patterns of early development and growth were studied. The period from fertilisation to 'first feeding' (i.e. when the yolk supply was almost exhausted) was described as a series of forty successive developmental 'steps'. A quantitative scoring system, whereby individual embryos were assigned points ranging from 1 to 1000 on the basis of assessment of numerous developmental features, was also developed. Trout reared at 10°C achieved greater values of developmental score and standard length, but lower values of body mass, from a given quantity of yolk compared to trout reared at 2°C. Yolk was used less efficiently for differentiation but more efficiently for growth in larger eggs. Although certain events, such as hatching, occurred relatively earlier at higher temperatures, the relative timings of most individual developmental changes, including myotube and muscle fibre formation and many aspects of neural development, did not vary with temperature. Muscle growth, particularly recruitment of new muscle fibres, was lower relative to developmental score and to length at 10°C than at 2°C. However, trout at first feeding of comparable total muscle area had significantly greater numbers of post-embryonic fibres when reared at the higher temperature, indicating the onset of a compensatory 'catch-up' in fibre recruitment. The effects of the migratory type of the female parent on development and growth of embryos and alevins were also investigated, but were found to be minimal. During a study examining the effects of forced exercise on growth, juvenile trout reared at 10°C as embryos exhibited a greater potential for somatic growth and recruitment of new muscle fibres than those reared at 2°C. It is postulated that this difference in growth potential is related to the higher numbers of myonuclei present in the 10°C-reared fish.

# Chapter 1: General introduction

What role does the environment play in shaping our offspring, as they grow and develop into independent, self-sufficient beings? The "nature versus nurture" debate is one that will doubtless continue among parents, teachers and sociologists for centuries to come. Any developmental biologist knows, however, that whatever its effects on human society, the environment is a powerful force in the life of every species on the planet.

The blueprints for development are provided by the parents, in the form of the genetic code; but this parental legacy is not the sole determinant of the phenotype of the offspring. The numerous processes whereby these DNA-coded instructions are converted into a living animal are often susceptible to the influence of environmental factors, such as nutrition, salinity, oxygen levels, and temperature. The latter is of particular importance to ectotherms such as fish, whose body temperature is determined by that of their surroundings. In these animals, environmental temperature can affect the complex processes of development from the moment the egg is fertilised. As a result, temperature can affect aspects of the animal's life such as the rate of growth, the final body size, and even the morphology and metabolism of individual tissues such as muscle. As much as 80% of the mass of a fish can consist of muscle tissue (Weatherley & Gill 1987), the biological machinery used to produce movement. As well as powering the swimming needed for prey capture, escape from predators, location of mates, and migration, muscle tissue can also act as a recycler of metabolites (Hulbert & Moon 1978), a nutrient supply during times of starvation (Beardall & Johnston 1985), and a source of heat (Block *et al.* 1994).

Fish are found in almost every aquatic habitat, ranging from the below-freezing waters of Antarctica to the geothermally heated lakes of East Africa, where water temperatures can reach 40°C (Lowe-McConnell 1987). Even within the estimated 20,000 species of bony fish (Class Telostei), various developmental programmes code for a dazzling assortment of forms, from the tiny Phillipine goby, *Pandaka pygmaea*, only half an inch long and believed to be the smallest of all vertebrates, to the bizarre ocean sunfish, *Mola mola*, which can weigh as much as 600 pounds (Migdalski & Fechter 1976). As well as occupying key niches in

practically every aquatic ecosystem on the planet, fish constitute a natural resource of tremendous economic importance; approximately 70 million tons are landed each year by commercial fishermen.

One of the most well known groups of fishes are those belonging to the Family Salmonidae, which are notable for their anadromous lifestyle. Although all members of the family spawn in fresh water, many of the species migrate to marine feeding grounds, where greater feeding opportunities lead to substantially improved growth rates. Subsequently, the fish return to their natal streams, homing with a high degree of accuracy (Stabell 1984). The trout (*Salmo trutta* L.) exhibits a broad range of life histories, from the brown trout which never leaves fresh water to the fully anadromous sea trout. The factors which determine the migratory type of individual trout are poorly understood. Individual populations are largely reproductively isolated (LeCren 1985), and may be adapted to the environmental conditions of their natal stream (Taylor 1991). Many populations of *S. trutta* are now in serious decline, particularly in the west of Scotland. It has been suggested that changes in the global climate may have consequences for aspects of salmonid biology such as the timing of migration and size at maturity (Mangel 1994). An increase in temperature as a result of global warming is also likely to affect developmental patterns, even during the embryonic period. Clearly, a greater understanding of the genetic and environmental factors affecting the development of trout is needed.

This thesis reports the results of investigations into the effects of temperature on the early development and growth of the trout (*S. trutta*), with particular emphasis on growth of muscle tissue. Comparisons have also been made between offspring of females with differing life history strategies. This introductory chapter reviews four areas of biology particularly relevant to these investigations.

The first section of this introduction, '**Building a fish**', describes the processes involved in the development of a newly fertilised egg into an independent, feeding fish. While many of these processes, particularly those that occur early on in the developmental programme, are highly conserved among fish species, a variety of reproductive strategies are available to fish, and these are described.

The second section of the introduction, '**Making muscle**', focuses on the development and growth of muscle tissue, from formation of the first muscle fibres in the embryo through to muscle growth in the adult fish. The various types of muscle, and the factors determining the potential for muscle growth in fish, are discussed.

In the third section, '**The importance of temperature**', some of the numerous ways in which temperature can affect life are described, as are some of the mechanisms fish have evolved in response to this important environmental variable. Particular attention is paid to the effects of environmental temperature on patterns of growth and development, including the plasticity of muscle development with temperature.

Finally, the fourth section, '**The trout, *Salmo trutta***', describes the life cycle and various life history strategies of trout. Current knowledge of the factors, both genetic and environmental, which may determine migratory type is reviewed. The general introduction concludes with a brief description of the experiments conducted in the present study, as presented in Chapters 2 to 6.

## Building a fish

### *Cleavage to gastrulation*

Following fertilisation, cytoplasmic movements divide the initially uniform zygote into an upper, cytoplasmic animal pole and a lower, yolk-rich vegetal pole – a process called bipolar differentiation. The cytoplasm at the animal pole of the egg concentrates into the blastodisc, which will ultimately give rise to the embryo proper. The blastodisc is separated from the surrounding eggshell, or chorion, by the perivitelline space (Armstrong & Child 1965). (Note: Most of the work carried out to date on early embryology of teleosts has been performed with reference to the zebrafish (*Brachydanio rerio* Hamilton-Buchanan); the findings of such studies are,

however, believed to be generally applicable to the early life stages of most fish species.)

During the period of cleavage, the blastodisc undergoes a series of divisions, without increasing in size, so that the resulting cells, or blastomeres, become progressively smaller. The first cleavage furrow divides the embryo vertically (i.e. meridionally), and then proceeds in a horizontal direction (Kimmel *et al.* 1990). The yolk itself is not divided during cleavage, that is, the cleavage is said to be 'meroblastic'. The next cleavage division also begins vertically, but at 90° to the first, resulting in four blastomeres in a 2 x 2 arrangement. Each cleavage division is highly synchronous - the mitotic divisions that turn the embryo from a 4-cell into an 8-cell embryo all begin at the same time, as do those that turn 8 cells into 16 cells, and so on (Kimmel *et al.* 1995). By the fifth or sixth division, blastomeres have begun cleaving horizontally. This results in a blastodisc which consists of two distinct populations of cells: the superficial 'enveloping layer' (EVL), and the 'deep layer' (DEL). The EVL gives rise to the periderm (Ballard 1966), a layer of epithelial cells surrounding the embryo which is lost prior to hatching (Bouvet 1976). DEL cells form the tissues of the embryo proper (Long 1984).

The cleavage period is followed by the blastula period, defined as beginning at the 128-cell stage in the zebrafish - although the teleost blastula differs from that of higher vertebrates in that it lacks a true blastocoel (Kimmel *et al.* 1995). During the early blastula period, the cell cycle lengthens irregularly, disrupting the synchronicity of the cleavage divisions. This occurs around the time of the eleventh cleavage division in zebrafish (Kimmel *et al.* 1995) and in the mummichog (*Fundulus heteroclitus* Walbaum) (Trinkaus 1992). The DEL cells at the bottom of the blastodisc fuse, to form the yolk syncitial layer (YSL) (Kimmel & Law 1985).

The gastrula period, which follows the blastula period, sees the onset of large-scale coordinated cell movements, termed 'morphogenetic movements', which lead to the formation of a true embryonic axis. Three main processes occur during gastrulation: epiboly, involution and convergent extension (Fig. 1.1). Epiboly occurs when the external margin of the YSL expands and moves downwards over the yolk towards the vegetal pole, dragging with it the rest of the blastodisc (Betchaku &

Trinkaus 1978). An increasing proportion of the yolk is thus enclosed by the blastodermal tissue.

The DEL cells at the leading edge of the expanding blastodisc undergo involution - while most of the blastodisc is moving downwards, these cells change direction and begin to move back upward, passing under the bottom of the other DEL cells (Morgan 1895; Wood & Timmermans 1988; Warga & Kimmel 1990). This causes the leading edge of the expanding blastodisc to thicken into the germ ring. The DEL cells which move back upwards under the blastodisc form the hypoblast, which eventually gives rise to the endoderm and mesoderm; the more superficial, non-involuting DEL cells form the epiblast, which produces the ectoderm (Morgan 1895).

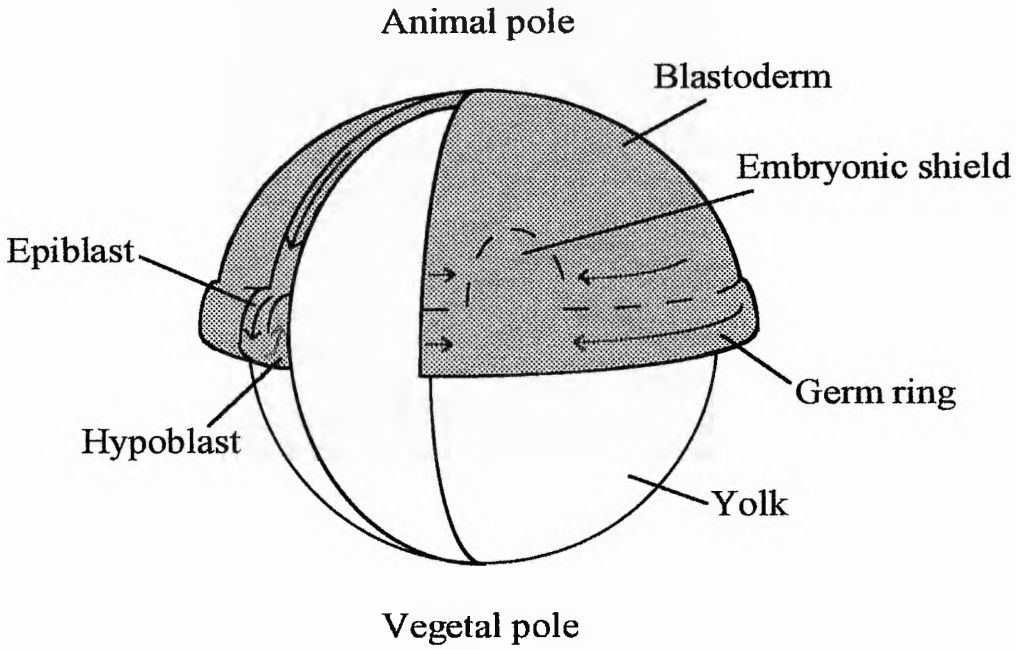



**Fig. 1.1.** Schematic of a typical fish gastrula, illustrating the main morphogenetic movements. Green = epiboly; red = involution; blue = convergent extension.

In addition to the changes wrought by epiboly and involution, the blastoderm is also reshaped by convergent extension (Oppenheimer 1936), which involves a general movement of cells in both hypoblast and epiblast towards one side of the egg (Ballard 1973a). These migrating cells converge to form a thickened region called the embryonic shield. As more cells accumulate, the shield extends towards the animal pole and resolves into the embryonic axis. Epiboly is completed when the germ ring reaches the vegetal pole and closes, so that the entire yolk is sheathed in blastoderm. This marks the end of gastrulation.

***Focus on:*** **Fate Maps**

During development, the various cells which constitute the early embryo become progressively allocated to increasingly precise lineages, each fated to form a particular organ, and then a particular tissue, until finally embryonic cells become committed to a specific cell type. In teleost embryos, the first such lineage restriction occurs early in development, with the compartmentation of the blastoderm into the DEL and the EVL (Kimmel *et al.* 1990). By the time gastrulation begins, almost all DEL cells are restricted to form specific tissues (Kimmel & Warga 1986). The fates of individual regions of the DEL can be mapped; techniques used have ranged from following the movements of coloured chalk particles inserted into particular regions of the embryo (Ballard 1973b), to injection of lineage tracer dye into single cells which can then be tracked with a high degree of accuracy (Kimmel *et al.* 1990). Oppenheimer (1936) produced one of the first fate maps for teleost embryos, for *Fundulus heteroclitus*. The most recent and accurate fate map for teleost gastrulae is that produced for zebrafish by Kimmel *et al.* (1990) (Fig. 1.2). It should be noted that this map includes sometimes extensive overlaps between adjacent regions, suggesting that the boundaries are not completely determined by the onset of gastrulation.

The fate map for teleosts illustrated in Fig. 1.2 follows a remarkably similar pattern to those previously produced for higher vertebrates, even down to the location of cells producing specific types of nerves (Kimmel *et al.* 1990), implying that the mechanisms of early developmental patterning have been highly conserved during vertebrate evolution.

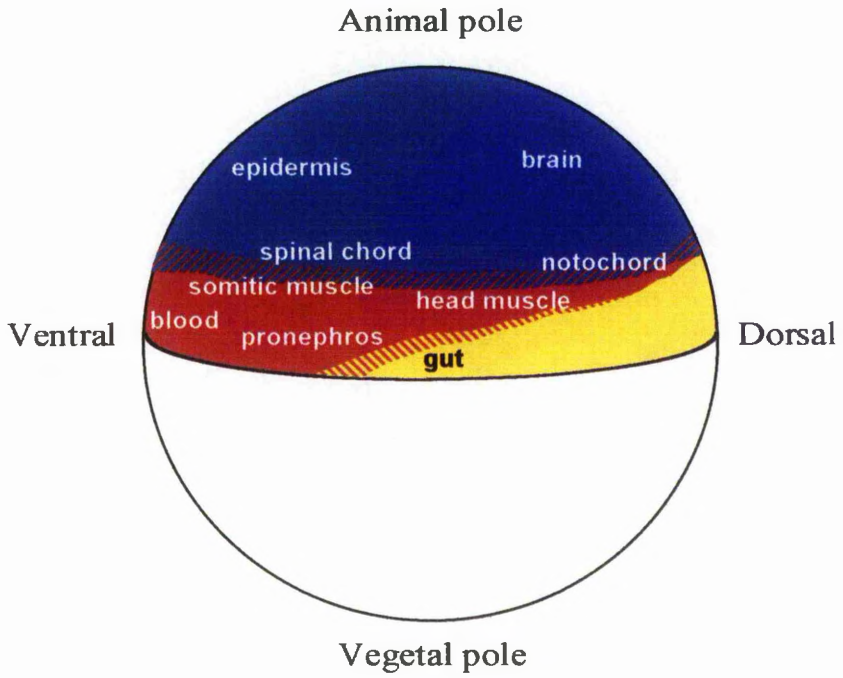



**Fig. 1.2.** Summary fate map for the zebrafish, at the beginning of gastrulation. Yellow: endoderm; Red: mesoderm; Blue: ectoderm. After Kimmel *et al.* (1990).

***Segmentation of the Body***

At the end of gastrulation, the component cells of the embryonic shield undergo complex rearrangements. The central region of the epiblast thickens as it begins to develop into the neural plate. The underlying hypoblast divides into central axial hypoblast, and flanking paraxial hypoblast, which will produce the somites (Kimmel *et al.* 1995). Somites are pairs of mesodermal segments, which will ultimately contribute to the vertebral column (Spratt 1955), the dermis, and the muscle blocks, or myotomes (Christ *et al.* 1978).

Segmentation has been defined as a "coincident repetition of elements belonging to most of the chief systems of organs along...the long axis of the body." (Bateson 1894, cited by Kimmel *et al.* 1991). While vertebrates are not as obviously segmented as, for example, annelids, the vertebrate body plan does show signs of repeating segments in the myotomes, the vertebrae, the pharyngeal or gill arches, and

in the nervous system. The first programming for segmentation may be acquired by cells as early as the gastrula stage (Kimmel & Warga 1986). The mechanisms which give rise to the original segmentation of the mesoderm into somites are not yet fully understood, however. Somite formation proceeds from anterior to posterior at a very regular rate (Gorodilov 1992), and it has been suggested that this rate is connected to the cell cycle. The time taken to produce a single somite pair is almost exactly the same as the time between successive cell divisions during the cleavage period (Gorodilov 1992), and approximately one-sixth to one-seventh of the cell cycle time during somitogenesis itself (Keynes & Stern 1988). Segmentation is also controlled by a number of homeobox genes (Njølstad *et al.* 1990), including genes of the *engrailed*-like *(eng)* family which are selectively expressed segmentally in the somites, rhombomeres (segments in the hindbrain), and the pharyngeal arches (Hatta *et al.* 1991). Such homeobox genes may regulate patterns of expression of genes such as *Her1*, which is expressed in alternating somite primordia in the zebrafish (Müller *et al.* 1996), although the function of this expression pattern is not yet known.

***Focus on:*** **Homeobox Genes**

Early studies on the fruit fly *Drosophila melanogaster* found that a mutation in a single gene could result in excess production of, or transformation of, entire body parts. For example, *antennapedia* mutants produce legs where they should have antennae. As the production of such complex body parts normally involves the activation of hundreds of genes, these mutations – known as homeotic mutations – must be affecting 'master' genes, whose activity regulates the activation of other, subordinate genes. A number of these homeotic genes were found to contain a highly conserved DNA sequence (McGinnis *et al.* 1984). This sequence, dubbed the 'homeobox', codes for a series of approximately 60 amino acids known as the 'homeodomain'; it enables proteins produced from homeobox-containing genes (e.g. transcription factors) to bind to DNA and regulate the expression of other genes. Genes with highly conserved homeobox sequences have since been identified in a whole range of organisms, including vertebrates; they play assorted key roles in embryonic development, helping to determine the different developmental capacities of various regions of the embryo (De Robertis *et al.* 1990).

***Formation of the organs***

The period of segmentation, or somitogenesis, also sees the establishment of most of the main organ systems within the fish embryo. While the precise dynamics of embryogenesis differ increasingly between fish species from this time on, the same fundamental processes occur during the somitogenesis period in most species. Kimmel *et al.* (1995) gives a highly detailed description of organ formation in the zebrafish; for a more general but basic model of fish embryonic development, see Timmermans (1987). For recent descriptions of development in salmonids, see Vernier (1969), which has drawings of exceptional quality, Ballard (1973c), Balon (1980) and Gorodilov (1983).

After the first somites are formed, the neural plate folds in on itself to produce the neural tube; the ventral midline of this structure is a specialised tissue called the floor plate, which itself plays an important role in the regulation of muscular and neural development (Yamada *et al.* 1993). The anterior-most portion of the neuroectoderm differentiates into the fore-, mid- and hindbrain, and the eyes and 'ears' of the fish begin to develop, in the form of the optic cups and otic vesicles. Beneath the neural tube, the supporting notochord forms, a stiff rod which will eventually contribute to the spinal column. The endodermal tissue, located ventrally within the embryo, forms the gut – first the intestine, then the stomach and liver - while between the notochord and the gut, paired pronephri, the future kidneys, appear. The fins begin to develop; the pectoral fins, and the embryonic median finfold running dorsally and ventrally down the midline, are the first to appear in zebrafish (Kimmel *et al.* 1995), *Fundulus* (Armstrong & Child 1965) and in the Atlantic salmon (*Salmo salar* L.) (Gorodilov 1983). With the production of functional muscle tissue in the somites, and establishment of muscle innervation, the embryo begins to move spontaneously.

One of the other major developmental events during the period of somite formation is the onset of circulation. The heart first appears as a pair of endocardial rudiments, located between the embryo and the yolk at the level of the hindbrain (Ballard 1973c), which then fuse to form a single vertical tube. At the same time, the 'intermediate mass', a peculiarity of teleost embryos (Swaen & Brachet 1899, cited by Isogai & Horiguchi (1997)), located between the notochord, pronephric ducts and

gut, begins to produce red blood cells (erythrocytes). When the first heart contractions begin, the circulatory system is normally limited to a simple loop, but by the time the last somites are being formed, the circulatory system usually begins to increase markedly in complexity. In embryos of some species, such as Atlantic salmon, the blood cells turn red as they acquire the haemoglobin required for oxygen transport (Gorodilov 1983), and blood vessels branch into the brain, the gill arches, between the myotomes, over the gut and into the tail. In addition, the yolksac becomes vascularised by a fine mesh of blood vessels. As well as serving to absorb nutrients from the yolk to fuel embryonic growth, these yolksac capillaries have also been attributed with a role in oxygen uptake (Holeton 1971), although Wells & Pinder (1996) reported that only 33% of the total $O_2$ uptake of salmonid embryos occurs at the yolksac, the rest being absorbed cutaneously directly into the embryonic body.

***Further development and hatching***

Among the remaining steps to be completed in the transformation from embryo to juvenile are formation of a functional mouth and gut, pigmentation of the eye to optimise visual ability and of the body for camouflage, formation and structural support of the full complement of fins, and development of a functioning branchial system for gaseous exchange, needed once the body exceeds the maximum size for cutaneous respiration alone (Hunt von Herbing *et al.* 1996). The timing of onset of these events is, however, highly variable, relative to two of the most important events in the life of a developing fish – hatching and first feeding. Although Balon (1990) assigned greater importance to the onset of exogenous feeding, hatching is perhaps the more visibly dramatic of the two changes. The emergence of the embryo from the chorion is achieved by intense thrashing movements, combined with the release of hatching enzymes that weaken the chorion. These enzymes are produced by holocrine cells, whose location varies from species to species; in salmonids, they are found on the head and anterior portion of the yolksac (Yokoya & Ebina 1976).

The degree of development of teleost embryos at hatch shows considerable variation between species (Timmermans 1987). For example, the tambaqui

(*Colossoma macropomum* Cuvier), a tropical species with small eggs, hatches in a very primitive condition, before the end of somitogenesis, lacking such basic structures as pectoral fins, jaws and eye pigment (Vieira & Johnston 1996). Salmonids, on the other hand, have large eggs rich in yolk, and are relatively highly differentiated at the time of hatching, possessing fully pigmented eyes, functional jaws, and all the major fins, as well as fully vascularised branchial arches with gill filaments (Ballard 1973c). The embryos of virtually all species hatch with a quantity of yolk supply still remaining, which fuels further growth and development during the 'eleutheroembryonic' or 'free embryo' period (Balon 1975b). One of the few exceptions to this rule is the diamond killifish (*Adinia xenica* Gilbert & Jordan), which hatches with little or no yolk but is ready to commence exogenous feeding immediately upon emergence from the chorion (Cunningham & Balon 1985).

***The larval period and reproductive strategies***

Those species whose embryos hatch in a relatively undifferentiated state usually include a larval period in their life cycle. Balon (1990) stated that "the main purpose of a larva is the acquisition of external nutrients when the endogenous supply is insufficient". The larvae of many species inhabit quite different niches (e.g. planktonic) than the adults, and may bear transient features designed specifically for the larval existence, such as extra respiratory structures, or spines and oil globules to improve buoyancy (Whittaker *et al.* 1973) (Fig. 1.3). The larval period, which can last a number of years, has been defined as the time from first exogenous feeding to the formation of the axial skeleton and complete differentiation of the fins (Balon 1975b). As well as being a time for acquisition of nutrients, the larval period can serve to improve dispersal (Barlow 1981), and to avoid predation or competition at the adult level.

The remodelling of the larval body into a juvenile form more closely resembling the adult is called metamorphosis; it can be metabolically expensive, and may result in a reduction in size, as in the viperfish (*Chauliodus sloani* Bean) (Belyanina 1977, cited by Balon 1986). For this reason, and also because of the great vulnerability of larvae to predators (Balon 1990), some species forego a specialised larval form, and instead have direct development - by the end of the free embryo

period, the body form approximates that of the adult. This is facilitated by increased maternal investment in the form of greater yolk reserves (Balon 1981; 1990), and is often combined with increased parental care, either in the form of active minding of the developing eggs, or 'hiding' strategies where the parents seek out locations with reduced danger of predation in which to lay their eggs (Balon 1975a). Salmonids have a greatly reduced larval stage; the onset of exogenous feeding practically coincides with the acquisition of the juvenile form (Smirnov 1975). The strategy of increased parental investment and reduced time spent in the larval period is taken to the extreme in mouth-brooders such as cichlids (Balon 1977), and species with internal fertilisation such as the coelecanth (*Latimeria chalumnae* Smith), the young of which can gestate for up to 13 months, achieving a weight of up to 800g before needing to feed exogenously (Wourms *et al.* 1988).

As well as reducing the length of the vulnerable larval period and avoiding the costs of metamorphosis, such a strategy usually has the additional benefit of greater offspring size at first feeding (Blaxter & Hempel 1963), which results in increased range in prey size and improved avoidance of predators (Miller *et al.* 1988). There are certain disadvantages, however - given a limit to the resources available to parent fish to invest in their offspring, larger egg sizes and greater parental care must therefore be associated with a compensatory reduction in the number of eggs produced. In addition, it has been suggested that larger eggs may have reduced survival pre-hatch due to a relative reduction in the surface area: volume ratio, which affects gas exchange (Garside 1959); and embryos inside the chorion can be just as vulnerable as larvae to predators. The balance between the advantages and disadvantages listed above depends on relative juvenile mortality rates (Sargent *et al.* 1987), and on the niche occupied by the species. Greater parental investment in individual offspring will tend to be favoured in stable, or *K*-selecting, environments, whereas a strategy of large numbers of offspring with little investment in individuals will be favoured in *r*-selecting environments, where conditions are unpredictable (Begon *et al.* 1996). Even within a species, egg size and number can vary substantially with age (Zonova 1973), stock densities (Anthony & Waring 1980, cited by Tanasichuk & Ware 1987) and environmental conditions such as temperature (e.g. Hempel & Blaxter 1967; Messieh 1976; Tanasichuk & Ware 1987).

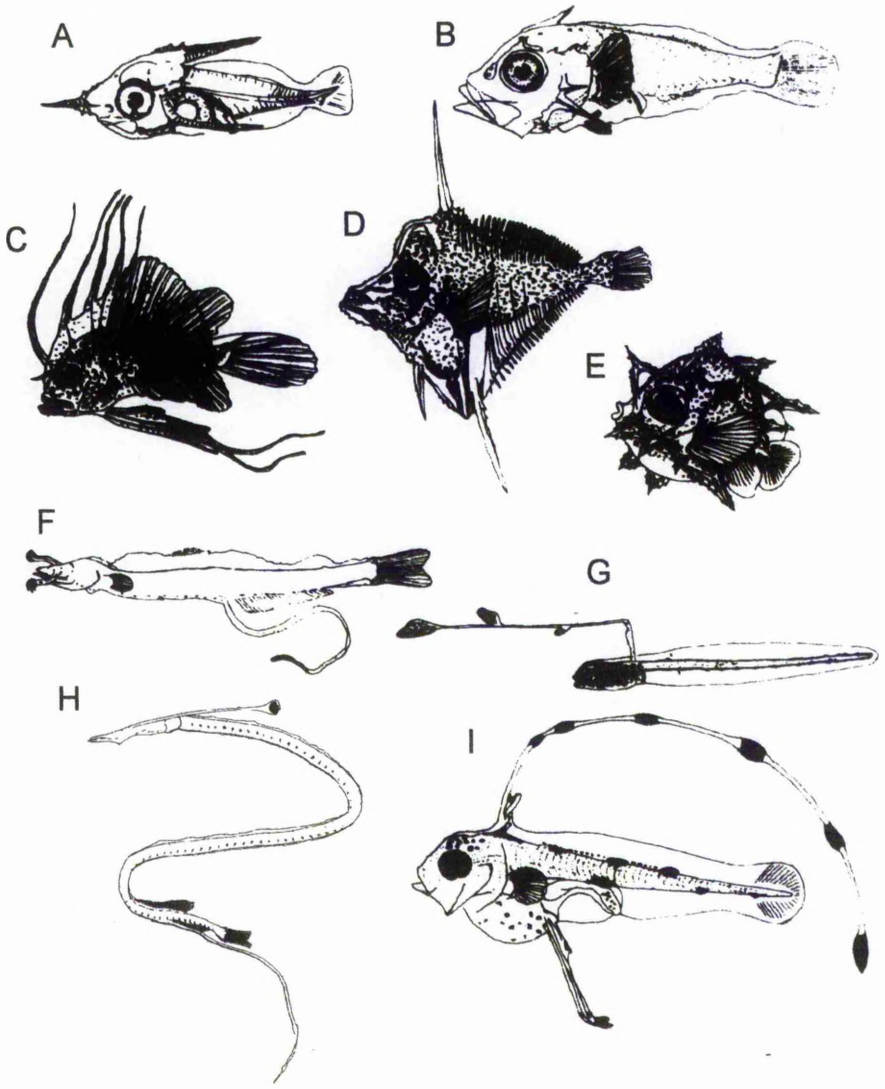



**Fig. 1.3.** Examples of fish larvae with spines and other adaptations to larval life. **A**: *Holocentrus vexillarius*. **B:** *Sebastes macdonaldi*. **C:** *Lophius piscatorius*. **D**: Acanthurid larva. **E**: *Ranzania laevis*. **F**: *Myctophum aurolaternatum*. **G**: *Carapus acus*. **H**: *Idiacanthus antrostomus* . **I:** *Zu cristatus*. **H** from Kawaguchi & Moser (1984), others from Blaxter (1988).

***Focus on:* Staging of Embryonic Development**

When studying any aspect of embryonic development, it is convenient to have an accepted system to describe a particular specimen's position in the ontogenetic sequence of that species. The terms 'embryo', 'free embryo' and 'larva' have been used above to distinguish young fish at different levels of development. For a more precise analysis of early development, a variety of systems have been used to split the embryonic and / or larval periods into further subdivisions. Probably the most common system is the 'staging series' (e.g. Armstrong & Child 1965, Iwamatsu 1994, Kimmel *et al.* 1995, Hill & Johnston 1997a), which involves describing the developmental progression as a series of discrete, sequential 'stages'. Each stage is based on a description of the embryo at an "instantaneous state of ontogeny" (Balon 1981). For example, a staging series prepared recently for the medaka (*Oryzias latipes*) divides the period from fertilisation to first feeding into 44 distinct stages; typical examples include stage 27, describing an embryo with 24 somites, and the following stage, stage 28, which describes a 30-somite embryo (Iwamatsu 1994). (Note: Somite numbers quoted usually refer to the number of bilateral pairs of somites.)

One of the drawbacks of such a system is that no provision is made for intermediate embryos which may display a mix of characters from two successive stages. This is avoided by the use of 'steps' rather than stages, as pioneered by Kryzhanovsky *et al.* (1953) & Vasnetsov (1953) (both cited by Balon 1990). A step is a *continuous* interval of ontogeny – modifying the example given above from the medaka staging series (Iwamatsu 1994), *step* 27 would include all embryos with 24 – 29 somites, describing the changes that occur during this interval.

A quite different method for quantifying the degree of differentiation of teleost embryos is the use of 'tau-somites'. A tau-somite ($\tau_s$) is the time taken to form one somite pair (Gorodilov 1996); although the rate of embryonic development, and thus $\tau_s$, varies with factors such as temperature and oxygen concentration, the value of $\tau_s$ as a proportion of the time to hatching remains constant (Gorodilov 1996). Therefore, if the age of the embryo is known it can be expressed as [Age / $\tau_s$], in units called 'somite intervals' (Gorodilov 1996). More simplified methods of

expressing embryonic age independently of developmental rate include the use of '% Time to hatch' (or '% Development time'), and 'degree-days' (the age of the embryo multiplied by the temperature), although the validity of degree-days as an indicator of development has been questioned (Kamler & Kato 1983).

## Making muscle

### *Structure of fish muscle*

The primary function of most muscle tissue is to contract and thereby to produce movement. Skeletal muscle tissue consists of large numbers of multinucleate muscle cells, or fibres, which contain myofibrils, the cellular structures that are responsible for this contractile activity. Each myofibril consists of a series of repeating units (sarcomeres), which are the source of the striations (I-bands and A-bands) visible in skeletal muscle fibres when examined under a light microscope. Each sarcomere, bounded by 'Z-discs' at either end, contains overlapping 'thick' filaments, consisting of molecules of the large (520 kDa) protein myosin, and 'thin' filaments, which contain actin (42 kDa), troponin and tropomyosin molecules (Bailey 1948) (Fig. 1.4). In the region of overlap, the thick and thin filaments are connected by cross-bridges extending from the myosin molecules (Huxley 1953). In addition, adjacent thick filaments are connected by a cross-linking structure known as the M-band (Luther *et al.* 1995), and sarcomeres also contain the structural molecule titin, the largest polypeptide yet described, which runs from the M-band to the Z-disc and is believed to serve to keep the thick filaments centred in the sarcomere (Horowits & Podolsky 1987).

Contraction of a muscle fibre is stimulated when neural input causes activation of voltage-sensitive proteins in invaginations of the muscle cell membrane; these invaginations, known as 'T-tubules', extend around each individual myofibril within the muscle fibre. The activated proteins trigger the release of $Ca^{2+}$ from the sarcoplasmic reticulum into the cytosol, which, mediated by the action of the accessory proteins troponin and tropomyosin (Zot & Potter 1987), stimulates contraction of the sarcomeres.

According to the 'sliding filament' theory, this contraction is achieved, not by shortening of the filaments themselves, but by the sliding of the thin filaments between the thick filaments, increasing the region of overlap (Fig. 1.4) (Huxley & Niedergerke 1954). The energy that drives this contraction is produced from the breakdown of ATP, catalysed by the myosin molecule (Engelhardt & Ljubimova 1939).

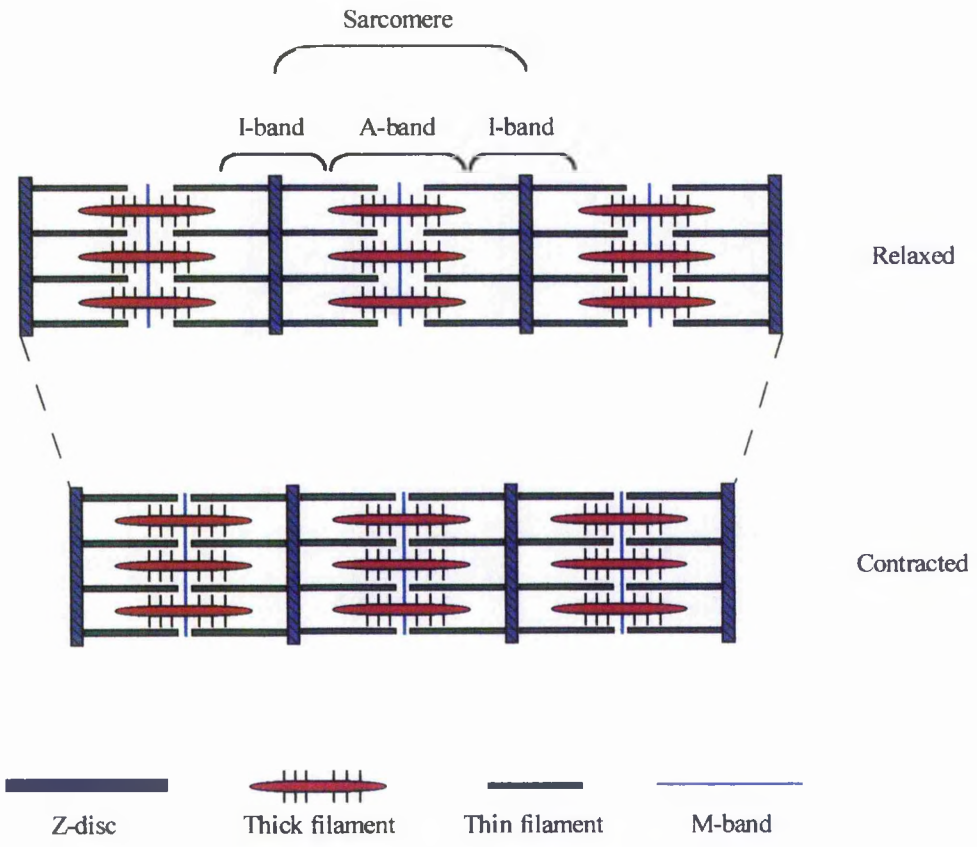



**Fig. 1.4.** Schematic diagram of a portion of a myofibril, illustrating the sliding-filament theory.

***The contractile phenotype of the muscle fibres***

As the requirements placed on a muscle vary in terms of strength, speed, and fatigue resistance, muscle fibres can exhibit a broad variety of contractile phenotypes. This can involve adaptation of virtually every aspect of the muscle; within a single animal, fibres specialised for different functions can express different isoforms of the contractile proteins (Hoh *et al.* 1976), can contain differing amounts or activities of the enzymes associated with energy production and myofibrillar contraction (Johnston *et al.* 1975), and can vary in the proportions of cell organelles such as energy-providing mitochondria and the sarcoplasmic reticulum (Nag 1972).

This wide range of potential fibre specialisations is usually categorised into a limited number of fibre 'types'. In the fish myotome, the two most important categories, known as 'white' and 'red' muscle, occur in distinct regions, which can be distinguished visually on the basis of colour. The white muscle makes up the bulk of the myotome in most fish species, particularly in more anterior positions (Nag 1972); in the rainbow trout (*Oncorhynchus mykiss*), white muscle makes up 87% of the total myotomal area posterior to the anus, and this figure reaches 99% anterior to the dorsal fin (Nag & Nursall 1972). The red muscle is most often present as a lateral strip of tissue, concentrated in a V-shaped region at the lateral midline (Fig. 1.5). In mammals and birds, the fibre types are not segregated into distinct zones as they are in fish muscle; instead, a single muscle can contain a mix of different fibre types, although the proportions vary depending on the muscle's role (Bagshaw 1982).

The red and white fibres differ not only in their location, but also in their diameter, ultrastructure, histochemistry and patterns of activation. The red muscle has a denser network of capillaries and greater concentrations of the oxygen-transport pigment myoglobin, which lends the tissue its darker colour (George 1962). Red fibres are normally smaller in diameter (Nag & Nursall 1972) and have higher densities of mitochondria than white fibres, but lower densities of myofibrils (Nag & Nursall 1972). Histochemical staining reveals that red fibres contain relatively more glycogen and lipids (Bone 1966), and higher activities of the oxidative enzyme succinate dehydrogenase, than their counterparts in the white muscle (Johnston *et al.* 1975). White fibres, on the other hand, have substantially greater myosin ATPase activity (Nag 1972), and also contain isoforms of contractile proteins, such as the

subunits of myosin, that are distinct from those of red fibres (Hoh *et al.* 1976). Many species of fish possess a third type of muscle, called intermediate or 'pink' muscle, which lies in a narrow strip between the red and white muscle tissues (Bone 1966). Pink fibres are intermediate in their size, myoglobin content and histochemical characteristics – although they can express greater activities of myosin ATPase than both red and white fibres (Johnston *et al.* 1975). As well as the red, pink and white muscle, tonic fibres, dedicated to maintaining body tone and attitude, have been identified in species such as the stickleback (*Gasterosteus aculeatus* L.) (Kilarski & Kozlowska 1983) and the plaice (*Pleuronectes platessa* L.) (Brooks & Johnston 1993). In muscle of the cod (*Gadus morhua* L.), seven distinct types of muscle fibre have been identified on the basis of their histochemical profile (Korneliussen *et al.* 1978).

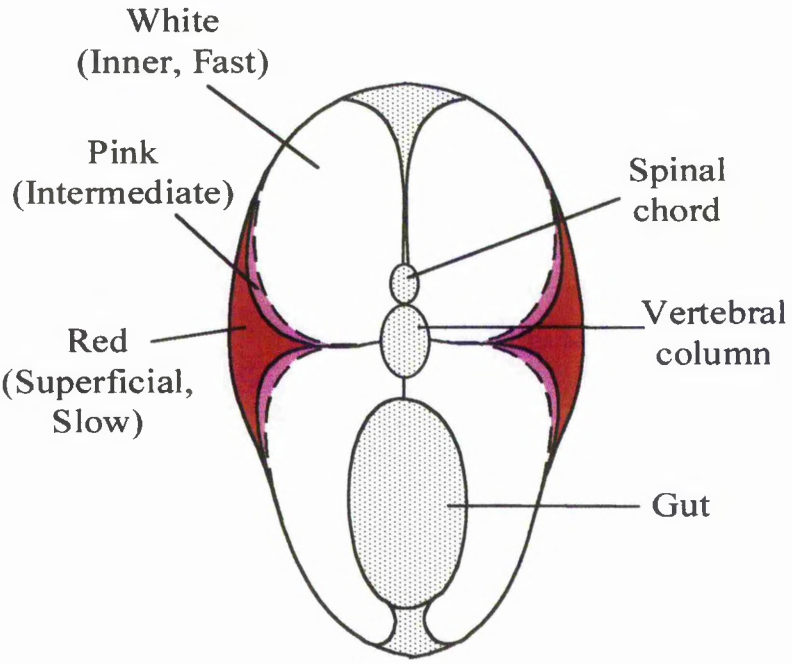



**Fig. 1.5.** Schematic diagram of a cross-section through a fish, illustrating the usual locations of the three main fibre types.

The structural differences between the fibre types are associated with their different functions, and this distinction in function is particularly strong in fish species. Red muscle, also called 'slow-twitch' muscle, is highly resistant to fatigue, and is generally used for continuous, or 'steady-state' swimming, which can be sustained indefinitely. White muscle ('fast-twitch') fibres are activated for rapid burst swimming, such as occurs during prey capture or the escape response (Bone 1966). White muscle can produce higher swimming speeds, associated with its greater myosin ATPase activity (Bárány 1967), but the rapid exhaustion of metabolic fuels and build-up of waste products from anaerobic metabolism (Duthie 1982) means that such swimming can only be maintained for short periods of time (Johnston *et al.* 1977). Fish whose lifestyle demands long periods of continuous swimming, such as mackerel, have relatively higher proportions of red muscle than those which rely more on darting movements separated by resting periods (Boddeke *et al.* 1959, Greer-Walker & Pull 1975). Exercise regimes that increase the levels of steady swimming activity often result in an increase in the amount of red muscle as a proportion of the total (Young & Cech 1993).

In fish species whose white muscle fibres are multiply rather than focally innervated (Barets 1961), white muscle may be activated, not just for high-speed burst swimming, but also for continuous swimming at intermediate speeds (Johnston *et al.* 1977). The threshold speed for activation of white fibres ranges from as little as 0.8 body lengths (BL).$s^{-1}$ in the saithe (*Pollachius virens* L.) (Johnston & Moon 1980a) to 3.6 BL.$s^{-1}$ in rainbow trout (Hudson 1973). Activation of pink fibres occurs at speeds which are higher than those which can be met by the red muscle alone, but not high enough to trigger activation of the white fibres (Johnston *et al.* 1977).

***Formation of the first muscle fibres***

Muscle differentiation begins in the more medial region of the embryonic somitic mesoderm, adjacent to the notochord (Waterman 1969). The undifferentiated myogenic cells (myoblasts) in this region form a particular subset called 'adaxial cells', about 20 in each somite. Even before the mesodermal tissue is segregated into somites – when it constitutes the 'lateral presomitic plate' - these adaxial cells can be distinguished in living zebrafish embryos with a light microscope, being larger and more regular in shape than the surrounding cells (Devoto *et al.* 1996).

When the embryo has 9 – 20 somites (depending on the species), a small number of the adaxial cells, usually two to six per somite, elongate to span the length of the somite, forming the uninucleate pioneer myotubes (van Raamsdonk *et al.* 1974). Pioneer myotubes show strong expression of homeobox *engrailed* genes, and may play a role in determining the future shape of the somite (Hatta *et al.* 1991). Production of myofibrillar proteins begins near the periphery of these myotubes (Vieira & Johnston 1996), and as the myotubes continue their differentiation into fully developed muscle fibres, they gain a striated appearance visible with a light microscope. This process begins simultaneously in the most anterior ten to twelve somites, and then proceeds caudally, somite by somite, at a regular rate (van Raamsdonk *et al.* 1974).

Coincidentally with, or shortly after, the formation of the pioneer myotubes, more lateral myoblasts begin to differentiate. These myoblasts do not elongate; instead, they line up in rows of three to six cells running parallel to the anterior-posterior (A-P) axis, and then fuse, under the control of genes such as the *rolling stone* gene identified in *Drosophila* (Paululat *et al.* 1995). The differentiation of the resulting multinucleate myotubes into muscle fibres, marked by the production of myofibrils, progresses in an anterior-to-posterior direction. Soon, the uninucleate pioneer muscle fibres are greatly outnumbered by their multinucleate counterparts (Waterman 1969).

***Genetic regulation of muscle development***

As with so many other aspects of development, formation of muscle tissue is under the extensive control of a number of homeobox genes. Members of the *Hox* family of homeobox genes may specify the identity of muscle cells along the anterior-posterior axis, as their *HOM* homologues do in *Drosophila* (Olson & Rosenthal 1994).

Signals from the neural tube and notochord, produced under the control of genes such as Sonic Hedgehog (*Shh*) and *Wnt* (Münsterburg *et al.* 1995), are also necessary for myotomal muscle development to occur normally (Rong *et al.* 1992, Blagden *et al.* 1997); the precocious development of the muscle pioneer cells has been attributed to their proximity to the notochord (Felsenfeld *et al.* 1991). Such

signals may be responsible for activation of the MyoD family (Olson & Rosenthal 1994), a group of muscle regulatory factors (MRFs) which control muscle formation in vertebrates. These DNA-binding basic-helix-loop-helix (bHLH) proteins include MyoD, myogenin, myf5 and MRF4 (also called Myf-6) (Buckingham 1994a; 1994b). The expression of any one of these genes in a normally non-myogenic cell can result in conversion to a myogenic pathway (Weintraub *et al.* 1991). Muscle-specific transcription factors bind to the DNA upstream of their target genes, promoting transcription. They bind in the form of heterodimers with more universally expressed bHLH proteins such as E12 and E47 (Buckingham 1994a); this heterodimerisation is mediated by the DNA strand itself (Wendt *et al.* 1998). Myogenesis can be negatively regulated by Id proteins, which sequester the bHLH muscle regulatory factors (Langlands *et al.* 1997).

Myf-5 is the first MRF to be expressed, even before somite formation, and is considered the 'upstream' gene that regulates the subsequent expression of the other MRFs (Buckingham *et al.* 1991). Myf-5 and MyoD are both believed to play roles in the commitment of undifferentiated mesodermal cells to a myogenic lineage, although they exhibit distinct expression patterns (Creuzet *et al.* 1998). Production of either Myf-5 or MyoD is necessary for the activation of myogenin and MRF4 (Buckingham 1994a; 1994b), which are now believed to regulate the expression of the individual genes responsible for the various processes of muscular differentiation - for example, the *fub-1*$^{+}$ gene, which plays a role in production and organisation of myofibrils (Felsenfeld *et al.* 1990).

The expression of genes of the MyoD family can also be regulated by thyroid hormone (Muscat *et al.* 1995), fibroblast growth factors and insulin-like growth factors (reviewed in Buckingham 1994*a*), and innervation patterns (Hughes *et al.* 1993). Expression patterns can also vary between different fibre types - MyoD appears to be of relatively greater importance in fast than in slow muscle, while the reverse is true of myogenin (Hughes *et al.* 1993). In addition to the MyoD family, other regulatory factors such as MNF, a 'winged-helix' transcription factor (Bassel-Duby *et al.* 1994), and myocyte enhancer binding factor 2 (MEF2) (reviewed in Buckingham 1994*a*, Ludolph & Konieczny 1995) are now increasingly considered as being of major importance in the early regulation of muscle development.

***Development of different fibre types***

There has been some debate over which of the fibre types – the inner white or the superficial red fibres – are formed first. Histological studies in several species have found that myoblasts deep within the myotome differentiate first to form the inner fibres, followed later by the formation of superficial fibres from more lateral myoblasts (Waterman 1969). In contrast, in the tambaqui, the superficial muscle consists of well-differentiated muscle fibres by the time of hatching, but the inner muscle is still at the myotube level of development (Vieira & Johnston 1996). Van Raamsdonk *et al.* (1984) also reported that superficial fibres differentiate before inner fibres in zebrafish embryos.

Recent experiments by Devoto *et al.* (1996) using immunocytochemistry and fluorescent labelling and tracking of individual cells suggest that those adaxial cells which do not form pioneer myotubes migrate laterally through the somite until they reach a superficial position, where they differentiate to form the first superficial muscle fibres. Production of slow-twitch-type myosin heavy chain subunits has begun in these cells even before they begin their migration. Blagden *et al.* (1997) suggested that the medial-to-lateral differentiation of non-pioneer fast fibres follows behind the migration of the slow muscle precursor cells in individual somites.

***Early innervation of the muscle***

Embryonic muscle fibres can be capable of contraction without neural stimulation (Whiting *et al.* 1992); such myogenic contractions may be propagated through the tissue by gap-junctions connecting the early myotubes and muscle fibres (Vieira & Johnston 1996). In zebrafish, spontaneous muscular contractions do not begin until the fibres have been innervated - the contractions are neurogenic, rather than myogenic (Grunwald *et al.* 1988). The fibres are innervated by the axons of primary motor neurons, the cell bodies of which are located in the neural tube (van Raamsdonk *et al.* 1984). The onset of neuromuscular transmission coincides with production of acetylcholinesterase, responsible for breakdown of the neurotransmitter acetylcholine released into the synaptic cleft during transmission (Katz & Thesleff 1957). Sensory nerves, such as Rohon-Beard neurons and the lateral line, also develop during the embryonic period (van Raamsdonk *et al.* 1984).

***Germinal zones***

When all the myogenic cells in the main mass of the somite have entered the phase of post-mitotic differentiation, recruitment of new inner white fibres becomes restricted to special germinal zones (Fig. 1.6a) (Stickland *et al.* 1988). These are regions of new fibre formation found at the dorsal, ventral and lateral extremities of the myotome. This embryonic pattern of fibre recruitment results in a decrease in mean fibre size from medial to superficial positions (Stickland *et al.* 1988). Additional red muscle fibres are added solely at the dorsal and ventral extremities of the superficial red layer, which in embryos is only one fibre in thickness (Brooks & Johnston 1993).

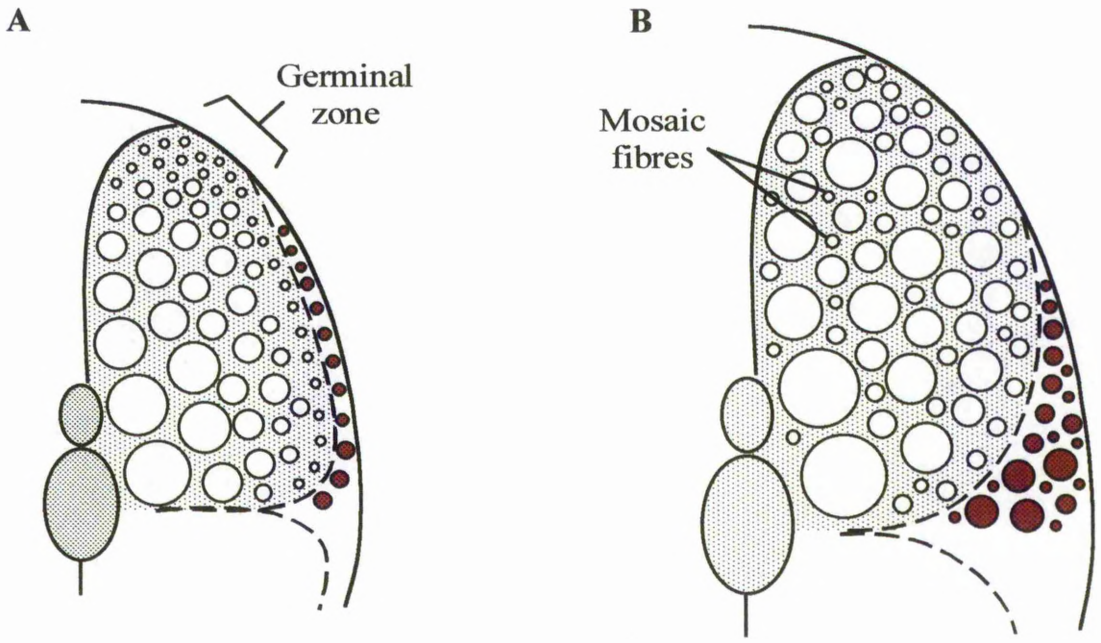



**Fig. 1.6.** Diagrams of the dorsal (epaxial) quadrant of a fish myotome. **A**: During the 'germinal zone' phase of muscle growth, all new fibres are produced at the dorsal and ventral apices of the myotome, and, in the case of the white muscle, at the lateral boundary. **B**: During the 'mosaic' phase of muscle growth, new fibres are produced throughout the myotome.

Although the origin of the myoblasts responsible for fibre production in the germinal zones has not been determined, Stoiber & Sänger (1996) suggested that these myoblasts may originate from the mesenchymal lining of the myotome. Whatever their source, they eventually become exhausted; in Atlantic salmon, this occurs shortly before first feeding (Johnston & McLay 1997). The fish then enters a third, postembryonic phase of muscle growth, which involves a special category of myoblasts called 'myosatellite cells'.

***The role of myosatellite cells in muscle growth***

Growth of muscle tissue requires an increase in the numbers of muscle nuclei. Fibre growth, or hypertrophy, requires the inclusion of new nuclei into the fibre to enable adequate control of the increased cellular volume (Cardasis & Cooper 1975). Production of new fibres (fibre recruitment, or muscle fibre hyperplasia) also requires nuclei. Muscle fibres are, however, completely post-mitotic – their nuclei cannot divide (e.g. Moss & Leblond 1971, Cardasis & Cooper 1975, Akster *et al.* 1995). Instead, once the germinal zones are exhausted, additional muscle nuclei are provided by myosatellite cells, which are capable of mitosis *in vivo* (Cardasis & Cooper 1975). First described by Mauro (1961), these spindle-shaped cells, with heterochromatic nuclei, little cytoplasm, and few organelles other than free ribosomes and polysomes, are usually situated between the cell membrane (sarcolemma) and the collagenous sheath (basal lamina) of established muscle fibres. In the early life stages of certain fish species, such as the European sea bass (*Dicentrarchus labrax* L.) and the Atlantic herring (*Clupea harengus* L.), myosatellite-like cells have been identified at stages prior to the formation of the basal lamina, and have been described as 'presumptive myosatellite cells' (Veggetti *et al.* 1990; Johnston 1993).

The myogenic nature of myosatellite cells has been demonstrated *in vivo* by labelling of replicating nuclei using [$^3$H]thymidine or the thiamine analogue BrdU (bromodeoxyuridine). Initially, myosatellite nuclei, but not muscle fibre nuclei, are labelled; subsequently, labelled nuclei can be seen aligned in rows, indicating the formation of myotubes (Johnston *et al.* 1998). Labelled nuclei also appear within mature muscle fibres (Moss & LeBlond 1970; 1971), and this can continue even after

the labelling reagent has been withdrawn; these nuclei must arise from myosatellite cells which have divided and then fused with existing fibres (Akster *et al.* 1995). Formation of myotubes and then muscle fibres from myosatellites has also been demonstrated *in vitro* (Powell *et al.* 1989). Ultrastructural studies have identified intermediate 'myosatellite fibres', cells similar in appearance to myosatellite cells but containing small amounts of myofibrils, and in the early stages of development of their own basal lamina (Akster 1983).

Studies on myosatellite replication in rats suggest that approximately 20% of the total number of myosatellite cells constitutes a reserve population; when cells in this population divide, one daughter cell normally remains in the reserve population, while the other daughter enters a 'producer' population, which constitutes the remaining 80% of the total myosatellite population (Schultz 1996) (Fig. 1.7). These producer cells undergo a limited number of further divisions (Schultz & Lipton 1982), with a shorter cell cycle time than that of the reserve cells (Schultz 1996), and then differentiate, either by fusing with existing fibres or by fusing with each other to form myotubes. The resulting new fibres are produced throughout the myotome; the mix of fibres of various ages and sizes has been described as a 'mosaic' appearance (Fig. 1.6b) (Boddeke *et al.* 1959).

The potential for new muscle growth is thus defined by the ability of the myosatellite population to generate new nuclei (Penney *et al.* 1983). This will depend on the size of the original reserve population; on the proportion of the reserve population which is dividing and contributing to the producer population; on the cell cycle time of both myosatellite types; and on the number of divisions which producer cells can undergo before becoming postmitotic and differentiating. The proliferative potential of myosatellite cells is known to decrease with the age of the animal, due to a reduction in the proportion of reserve cells that replicate and an increase in the cell cycle time (Schultz & Lipton 1982).

Although most work on postnatal / postembryonic muscle growth in vertebrates now assumes that myosatellite cells are solely responsible for production of new nuclei, Koumans *et al.* (1994) calculated that, in carp, the number of myosatellite cells present is insufficient to account for the rate of production of new

muscle nuclei, particularly in larger fish. The myotome therefore may contain additional myogenic cells other than the myosatellites, outside of the basal laminae of the fibres (Koumans & Akster 1995).

***Fibre recruitment and ultimate body size***

In birds and mammals, recruitment of new fibres only plays a substantial role in postnatal muscle growth in the first month of life after birth or hatching, and during periods of rapid muscle growth due to intensive exercise (Gonyea *et al.* 1986) or regeneration of muscle tissue after injury (Campion 1984). In contrast, many fish species maintain production of new muscle fibres for a significant part of their life after hatching, resulting in substantial increases in the total number of fibres (e.g. Greer-Walker 1970, Stickland 1983, Romanello *et al.* 1987, Kiessling *et al.* 1991, Rowlerson *et al.* 1995). A first-feeding salmon free embryo (or 'alevin'), whose germinal zones have recently been exhausted, typically possesses 8,000 - 10,000 white muscle fibres (Johnston & McLay 1997). By the time it has reached a length of 60cm, the activity of the myosatellite cells will have increased this number to 800,000 or more (I.A. Johnston, pers. comm.).

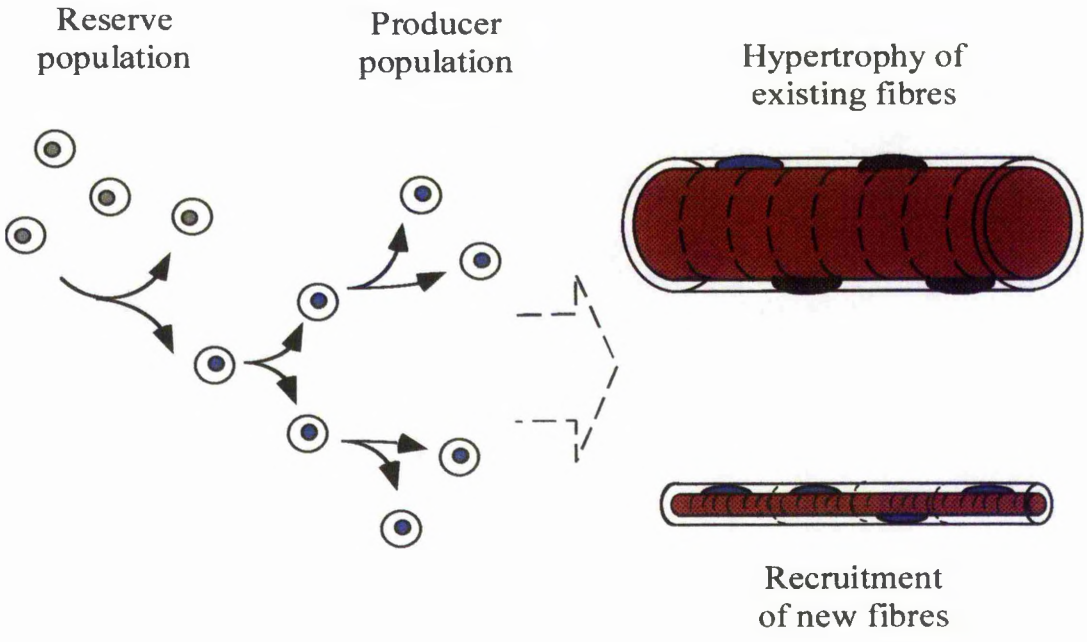



**Fig. 1.7.** Schematic illustrating the dynamics of production, by myosatellite cells, of new myonuclei, based on the findings of Schultz (1996).

There is a limit to the maximum size (and therefore, surface area : volume ratio) a fibre can reach, above which sufficient diffusion of oxygen and metabolites becomes impractical. Weatherley *et al.* (1988) suggested that the upper limit to fibre size, which is probably species-specific (Weatherley & Gill 1985), lies in the range of 120 - 270μm, although white muscle fibres up to 450μm in diameter have been found in the Antarctic fish (*Notothenia neglecta* Nybelin) (Battram & Johnston 1991). The total growth a fish can achieve is ultimately defined, not only by the maximum size of the fibres, but also by its ability to recruit new fibres to the myotome. Earlier termination of fibre recruitment is linked to smaller final body size, both between species (Weatherley & Gill 1985), and between sexes (Calvo 1989) or differing migratory types within species (Meyer-Rochow & Ingram 1993). Typically, production of new fibres is halted when a fish has reached approximately 44% of its maximum body size (Weatherley *et al.* 1988). The phase of 'mosaic' muscle growth may even be completely absent from slow-growing species, such as the guppy (*Poecilia reticulata* Peters), which never attain large body sizes (Veggetti *et al.* 1993).

*Focus on:* **Analysis of Muscle Cellularity**

There are two fundamental processes contributing to muscle growth - recruitment of new fibres, and increase in size of existing fibres. The relative importance of each process to the achievement of a given quantity of muscle varies both between species (Weatherley *et al.* 1988) and between families and age groups within species (Luquet & Durand 1970), and is influenced by environmental factors such as temperature. It has been suggested that the two processes are independently regulated, and may even be 'in competition' with each other for available nuclei (Rowlerson *et al.* 1995).

Fish which have relied to a relatively greater extent on fibre recruitment during their muscle growth will have greater fibre numbers at a given size, while fish for which fibre hypertrophy has been of relatively greater importance will have greater mean fibre sizes (Fig. 1.8). The relationship between muscle fibre number and fibre size is known as the 'muscle cellularity'. Fibre size can be measured with

equal validity as either the cross-sectional area of the fibre (e.g. Higgins & Thorpe 1990, Johnston 1993, Hanel *et al.* 1996), or as the equivalent diameter, calculated assuming the fibres to be circular in cross-section (Stickland 1983).

In small fish, such as embryos and larvae, fibre numbers, and the sizes of all the individual muscle fibres, can be measured relatively easily from histological sections (Johnston 1993). This becomes impractical in larger specimens (e.g. a salmon with 800,000 fibres), and so estimates must be made. Mean fibre area can be estimated based on measurements made from one or more representative areas within the myotome; the minimum number of fibres considered necessary for an accurate estimate ranges from 15 (Weatherley & Gill 1984) to 300 (Stickland 1983). This value can then be divided into the value of total cross-sectional area of the muscle, to produce an estimate of fibre number (Stickland 1983). Alternative methods for measurement of fibre number include digestion of an entire muscle with nitric acid followed by visual counts of the dissociated fibres (reviewed in Antonio & Gonyea 1993). Fibre areas have even been measured by passing dissociated fibres, trimmed to fragments of equal length, through a Coulter counter (Layman *et al.* 1980).

The value of the mean size of the muscle fibres has often been used as a measurement of the contribution of fibre hypertrophy to muscle growth (e.g. Willemse 1976, Stickland 1983), but recently Johnston *et al.* (1998) have chosen to use the mean area of just a subset of the fibres (e.g. the largest 200) as an indicator of hypertrophy. This is because the value of mean fibre size is affected, not just by fibre growth, but also by the levels of recruitment of new (and therefore, small) fibres to the myotome. Two fish with equal rates of fibre hypertrophy but differing rates of fibre recruitment may have relatively similar total amounts of muscle (as the new fibres as yet make only a minor contribution), but quite different values of mean fibre size (Fig. 1.9). The mean size of just the largest, and presumably, oldest, fibres is unaffected by rates of fibre recruitment, however. Histograms illustrating the frequencies of fibre sizes have also been used to examine patterns of muscle growth (Willemse 1976). When it is considered that rates of both fibre recruitment and hypertrophy can vary substantially during the life of a fish, analysis of changes in muscle cellularity can become quite a complex matter.

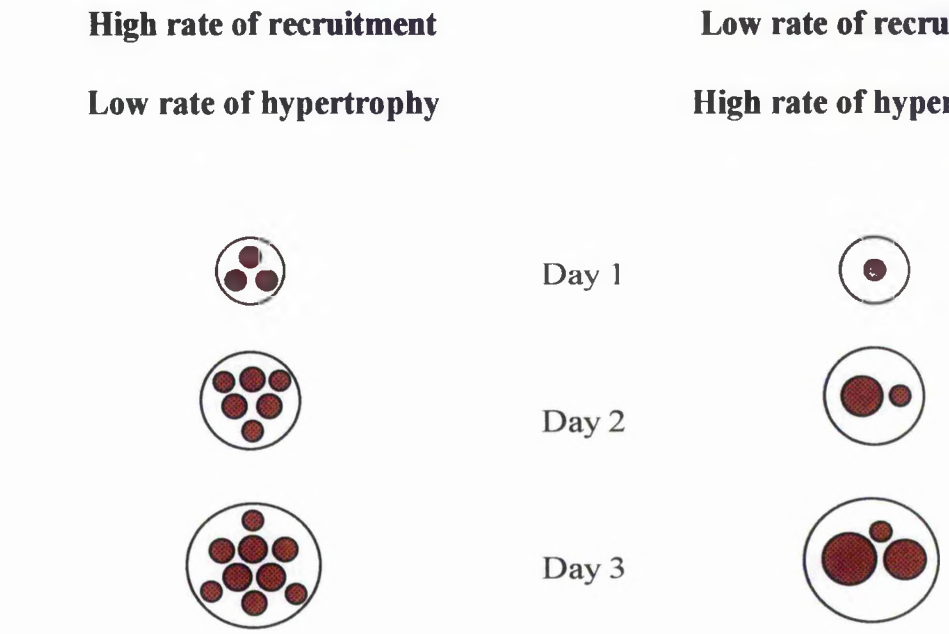



**Fig. 1.8.** Schematic illustrating hypothetical muscle growth in two fish with differing rates of fibre recruitment and fibre hypertrophy. The filled red circles represent muscle fibres in cross-section. Although the total area of muscle produced by day 3 is identical in the two fish, the values of fibre number and mean fibre size differ markedly i.e. there are differences in the muscle cellularity.

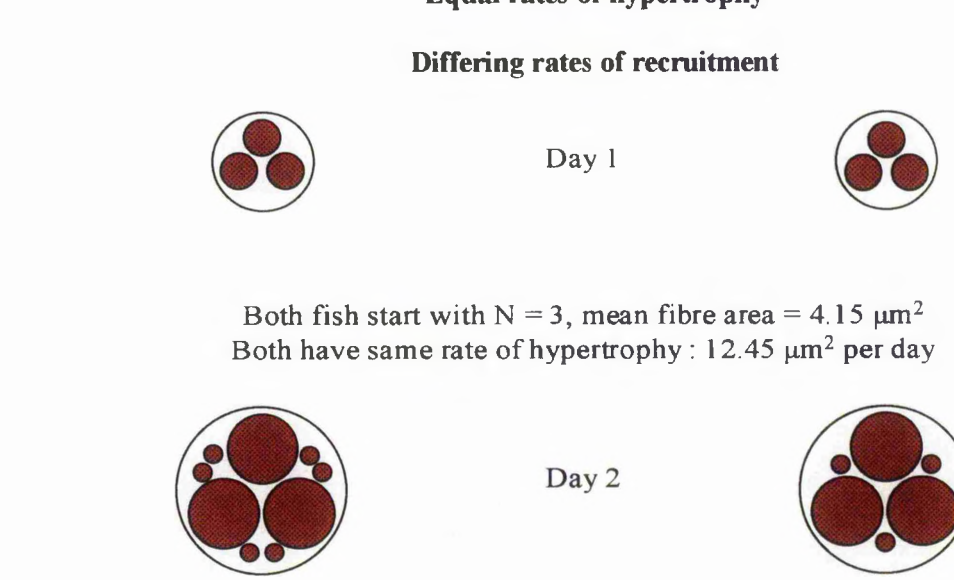



**Fig. 1.9.** Schematic illustrating hypothetical muscle growth in two fish with identical rates of fibre hypertrophy, but differing rates of fibre recruitment. By day 2, the total muscle area is only 5% lower in the fish with a low rate of recruitment. But, although rates of fibre hypertrophy are the same in the two fish, the value of mean fibre area in the low-recruitment fish is 40% higher than that of the high-recruitment fish. The rate of hypertrophy is more accurately represented by the value of the mean area of the largest 3 fibres, which is identical in the two fish.

## The Importance of Temperature

Temperature is probably the most critical of all the environmental variables which affect an animal's life. A quick examination of studies from recent years produces findings of temperature effects on systems as diverse as community structure of zooplankton (Moore *et al.* 1996); growth performance in pigs (Panagakis & Kyritsis 1991) and digestion patterns in sheep (Weniger & Stein 1992); transmission rates of the salmon parasite *Gyrodactylus salaris* Malmberg (Bakke 1991) and regulation of diapause in insect pests (Bell 1994); triggering of 'ballooning' behaviour in spiders (Weyman 1993); yolk synthesis in lobsters (Quackenbush 1994); prey selection by lizards (Diaz 1995); and social interactions in grey seals (Caudron 1997).

### *Thermal tolerance ranges*

Ectotherms, including most fish, cannot regulate their body temperature independently of that of the external environment in the way that endotherms such as birds and mammals can, and so are particularly vulnerable to both short- and long-term changes in temperature. Obviously, the most fundamental impact any environmental variable can have on life is to end it. All species have thermal tolerance ranges within which they can maintain the metabolism and behaviour required to survive. Exposure to temperatures outside this range is fatal, due to factors such as disruption of cells by ice crystals, heat inactivation of enzymes, differential temperature effects on interdependent metabolic reactions, and changes in membrane structure (Schmidt-Nielsen 1975). Naturally, species have adapted their normal thermal tolerance ranges to match the environment in which they have evolved. Antarctic notothenioid fishes can survive below 0°C, but temperatures above 6°C are fatal to them (Somero & DeVries 1967); in contrast, the intertidal goby (*Gillichthys seta* Ginsburg) has a thermal range that is both shifted to a higher point on the temperature scale, and much broader, being able to survive at temperatures between 8 and 40°C (Dietz & Somero 1992). Although 50°C appears to be the absolute upper limit above which no member of the animal kingdom can

complete its life cycle (Schmidt-Nielsen 1975), certain thermophilic bacteria can thrive at temperatures above 100°C (Brock 1985).

The thermal range of a particular species often changes during its life. Adults usually have broader thermal ranges than embryos or larvae, making them more resistant to temperature extremes (Schmidt-Nielsen 1975). Embryos of the trout (*S. trutta*) can only survive at temperatures between 0°C and 15°C, but adults can withstand temperatures as high as 30°C (Elliott 1994).

***Acclimatisation***

Within the thermal tolerance range of a given species, temperature has major effects on the metabolic rate, which usually increases two- to threefold with an increase of 10°C - in other words, the $Q_{10}$ of metabolic rate normally lies between two and three (Schmidt-Nielsen 1975). However, often when animals are maintained at low temperatures for a period of time they can adjust their metabolism to compensate somewhat for the effects of temperature - they become 'acclimatised'. (Note: 'Acclimatisation' is used to describe changes in response to natural conditions; the term 'acclimation' refers to changes in response to experimental conditions produced in the laboratory, where temperature alone is varied (Schmidt-Nielsen 1975)). An example of this kind of metabolic compensation is a study by Armitage & Lei (1979), working with the water flea (*Daphnia ambigua* Scourfield). The metabolic rate of 10°C-acclimated *Daphnia*, measured at 10°C, was only slightly lower than that of 20°C-acclimated *Daphnia*, measured at 20°C (Fig. 1.10). When both acclimation groups were compared at an identical temperature, the cold-acclimated fleas had substantially higher metabolic rates than the warm-acclimated fleas, due to acclimatory compensation.

As well as metabolic rate, temperature can affect other phenotypic aspects such as swimming performance, and so some species have also evolved acclimation responses in these functions. Fry & Hart (1948) showed an acclimatory response of swimming speed to temperature by the goldfish (*Carassius auratus* L.). The thermal range for locomotor activity also differed between the acclimation groups (Fry & Hart 1948). Changes at the cellular level which enable acclimation in swimming

ability include increased activity of myosin ATPase in cold-acclimated fish (Johnston *et al.* 1975; Hwang *et al.* 1990), and production of different isoforms of myofibrillar proteins at different temperatures (Goldspink *et al.* 1992; Imai *et al.* 1997; Watabe *et al.* 1998). Acclimatisation responses can also involve changes at the tissue level, such as the increase in the proportion of red muscle found in goldfish maintained at low temperatures (Johnston & Lucking 1978); this compensates for the reduction in power output per unit mass of muscle which occurs as temperature decreases.

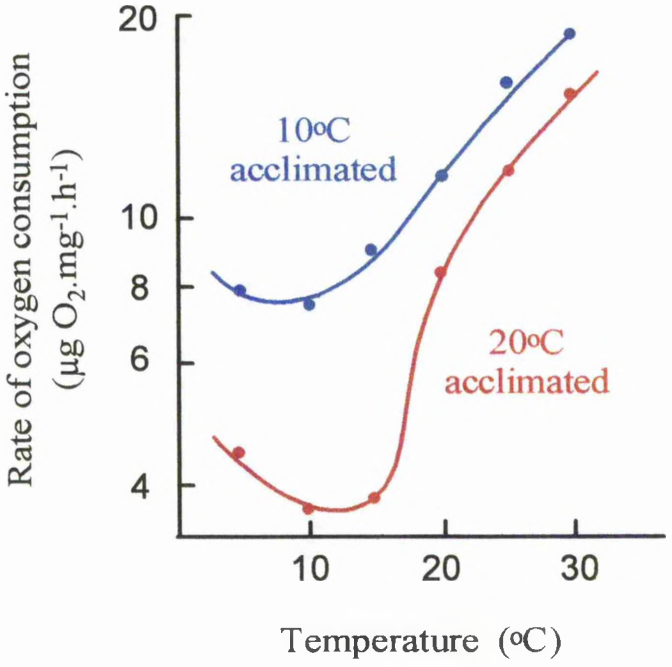



**Fig. 1.10.** Acclimatory compensation in oxygen consumption of the water flea *Daphnia ambigua* (After Armitage & Lei 1979).

***The basis of phenotypic plasticity***

The examples of acclimation listed above demonstrate that the phenotype of an organism is not determined solely by the genotype; it can also be modulated in response to environmental variables such as temperature. This phenomenon is called 'phenotypic plasticity' - "the change in the expressed phenotype of a genotype as a function of the environment" (Scheiner 1993). The fundamental mechanisms behind

such plasticity are not yet fully understood. Proposed 'pleiotropic' models involve straightforward differential expression of given genes under different environmental conditions - for example, heat-shock genes, which produce proteins important for the development of cellular thermotolerance, are only expressed in response to sublethal heat shock (Li & Werb 1982). Combinations of genes may also be affected differentially by temperature, resulting in alterations in phenotype. Alternatively, 'epistasis' models describe one set of genes which determine the average phenotypic expression of an animal, while separate genes, affected by temperature, control the magnitude of phenotypic response relative to that average (Scheiner 1993). There may be costs associated with the maintenance of the genetic and cellular machinery necessary for phenotypic plasticity, and so it is usually more likely to be favoured under conditions of environmental heterogeneity.

***Temperature and rates of growth and development***

Within the thermal tolerance range of a given ectothermic species, temperature has major effects, not only on the metabolic rate, but also on rates of development and growth, which increase substantially with increasing temperature. Consequently, fish embryos generally hatch after much shorter periods of time when reared at higher temperatures (Fig. 1.11).

Interestingly, differences in growth rates between related organisms from different latitudes are not always as great as might be expected, given the differences in environmental temperatures they experience (Cossins & Bowler 1987). It appears that, at least in some species, certain populations have evolved 'latitudinal compensation' (also known as 'countergradient variation') in growth rates - the reductions in annual growth achieved by populations at higher latitudes, due to lower temperatures and shorter growing seasons, may be somewhat compensated for by higher intrinsic growth rates (Gerking 1966). Conover (1990) examined growth in populations of three fish species - the American shad (*Alosa sapidissima* Wilson), the striped bass (*Morone saxatilis* Walbaum) and *Fundulus heteroclitus*; he found that although the length of the growing season declined by a factor of about 2.5 with increasing latitude in each species' range, total growth achieved in the first year was comparable between northern and southern populations.

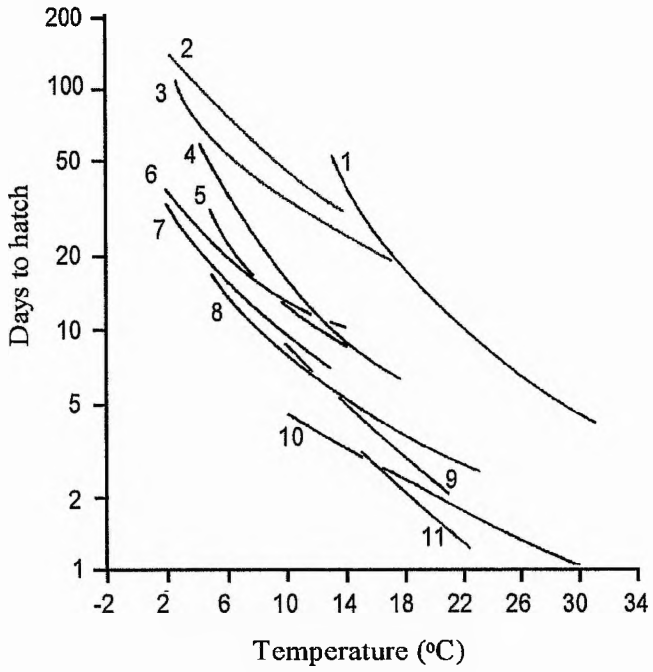



**Fig. 1.11.** Graph of time from fertilisation to hatching against temperature, for 11 fish species. Curves for salmonid species are illustrated in blue. 1: Desert pupfish *Cyprinodon macularius*. 2: Brook trout *Salvelinus fontinalis*. 3: Rainbow trout *Salmo gairdneri* ( = *Oncorhynchus mykiss*). 4: Smelt *Osmerus eperlanus*. 5: Atlantic herring *Clupea harengus*. 6: Plaice *Pleuronectes platessa*. 7: Pacific cod *Gadus macrocephalus*. 8: Rockling *Enchelyopus cimbrius*. 9: Mackerel *Scomber scombrus*. 10: Grey mullet *Mugil cephalus*. 11: Striped bass *Morone saxatilis*. After Blaxter (1988).

***Temperature and the relationship between growth and development***

In addition to the effects of temperature on growth and development rate, it has long been known that higher temperatures during development usually result in reductions in size at maturation, both between and within species (Ray 1960). Although exceptions do exist (e.g. Lamb & Gerber 1985), Atkinson (1994) found that of 109 studies, on species from nine different phyla including protists, plants and animals, 91 studies reported a negative correlation between temperature and adult

body size. Geographical clines in body size (Partridge & French 1996) usually have at least a partial genetic basis resulting from natural selection (Endler 1977, cited by Partridge & French 1996), but there is also substantial evidence of phenotypic plasticity of adult body size with temperature (Ray 1960, von Bertalanffy 1960). Temperature-induced changes in size occur, not just in adults, but also at earlier times during development; even embryos are usually smaller at a given level of development when reared at higher temperatures (Bengtson *et al.* 1987).

Von Bertalanffy (1960) argued that this dissociation between growth and development could be due to a greater acceleratory effect of temperature on catabolic processes than on anabolic processes - although it has since been argued that such an explanation would also be expected to produce slower, not faster, growth at higher temperatures (Atkinson 1994). Alternatively, constraints on growth may be exacerbated at higher temperatures (Atkinson 1996). It is known that, within the thermal tolerance range of a species, relatively high environmental temperatures can be associated with reductions both in the quantity of yolk supplied to each egg (Blaxter & Hempel 1963) and in the efficiency with which the yolk supply is converted into embryonic tissues (Hayes *et al.* 1953). Reduced efficiency may be due to greater expenditure of resources on metabolic activity, leaving less available for growth (Hamor & Garside 1979). However, there have been cases of improved yolk utilisation efficiency at higher temperatures (Kamler & Kato 1983). In addition, oxygen concentration, and the size of the egg, can also affect the efficiency of conversion of yolk into tissue (Kamler & Kato 1983).

***Plasticity during Development***

Plasticity can occur at any life stage, but is often most powerful during the early development of an animal, when its structures are first being formed. This is 'developmental plasticity', which is now believed to be an important mechanism of evolutionary change. Sharov (1966, cited by Balon 1990) stated that "Evolution of living beings is a process of change in all the stages of individual development. (New phenotypes) evolve by introducing ... changes into the developmental program of early ontogenetic stages, which are more plastic than later ontogenetic stages." The effect of temperature on the relationship between growth and development, described

above, is a simple example of such developmental plasticity. A more extreme example is exhibited by colonies of the ant *Pheidologeton diversus* Jerdon, which have four different subcastes of worker; the subcaste joined by individual larvae is determined, not genetically, but entirely by the conditions under which they are reared (Holldobler & Wilson 1990, cited by Scheiner 1993). In ectothermic vertebrates, the gender of the adult can be partially or completely determined by the temperature experienced by the embryo (DeSouza & Vogt 1994).

***Focus on:*** **Meristic characters**

A common example of developmental plasticity is variation in meristic characters - "serially repeated structures such as vertebra or fin rays" - in response to environmental variables (Lindsey 1988). The number of such characters can be quite flexible during the embryonic period, but then becomes fixed (Lindsey 1988). Jordan's rule states that fish from polar or cooler waters tend to have greater numbers of vertebrae than relatives from tropical or warmer waters, and holds true for many different fish groups at different taxonomic levels. Vertebral number across freshwater species (relative to body length) also increases with increasing altitude, and the critical factor appears to be environmental temperature (Lindsey 1988). Differences between populations at different latitudes are at least partially genetically determined (Billerbeck *et al.* 1997), but even among genetically identical offspring, the range of vertebral numbers produced can differ without overlap between groups reared at different temperatures (Harrington & Crossman 1976). It has been proposed that such plasticity of meristic traits is a consequence of differential temperature effects on development and growth - if individual meristic characters e.g. a single vertebra, are fixed in size, but the total body size is relatively reduced at higher temperatures, then that character will be produced in lower numbers due to size limitations (Lindsey 1988).

***Temperature and the Relative Timing of Developmental Changes***

Temperature affects the overall rate of development of an ectothermic organism; but the developmental sequence as a whole contains a wide variety of programmes of differentiation for the various organs and tissues being produced, and these programmes may have different temperature sensitivities. As a result, the precise order in which structures are formed may depend on environmental temperature. Hayes *et al.* (1953), studying embryonic development in the Atlantic salmon, found that in embryos reared at high temperatures (e.g. 10°C), bile production in the gut began prior to the completion of vascularisation of the yolksac and establishment of circulation in the caudal fin. However, when embryos were reared at low temperatures (e.g. 3.5°C), bile production was delayed relative to the two circulatory features, so that they appeared first, a reversal of the order seen at 10°C. It was suggested that high temperatures favoured the development of the fins, pigmentation and the circulatory system, relative to overall development, while low temperatures promoted hatching and differentiation of the digestive system and skeleton (Hayes *et al.* 1953). Such shifts in the relative timing of individual developmental changes as a result of different environmental temperatures have since been reported in a number of other fish species (Fukuhara 1990, Johnston 1993). Extreme heterochrony may result in gross abnormalities such as Siamese twins, which are more common when embryos are reared at temperature extremes, particularly high temperatures (Hayes *et al.* 1953).

***Temperature and Muscle Development***

The thermal regime experienced by fish embryos and larvae can have major consequences for their patterns of muscle formation. Using the number of somites in the embryo as an indicator of the level of overall development, Johnston *et al.* (1997) showed that production of myofibrils, and synthesis of acetylcholinesterase at the neuromuscular endplates, began relatively earlier in herring embryos reared at 15°C than in embryos reared at 12°C or lower. Outgrowth of axons from the primary motor neurons is also relatively delayed at lower temperatures (Hill & Johnston 1997b), as is differentiation of red and white muscle fibre types in herring embryos (Johnston *et al.* 1995), but not in Atlantic salmon embryos (Usher *et al.* 1994). During larval development of herring, the independent regulatory processes for

different myofibrillar protein isoforms (Pette & Staron 1990) are differentially affected by temperature. As a result, unique combinations of the various isoforms occur in larvae reared at different temperatures (Crockford & Johnston 1993; Johnston *et al.* 1997). At the ultrastructural level, herring larvae reared at 15°C have higher volume densities of mitochondria in their muscle fibres than larvae reared at 5°C (Vieira & Johnston 1992) - the reverse of the situation found when comparing warm- and cold acclimated crucian carp (*Carassius carassius* L.) (Johnston & Maitland 1980). Volume densities of myofibrils in the fibres are significantly lower at 15°C in the red fibres, but not in the white fibres (Vieira & Johnston 1992).

The rates of fibre recruitment and fibre hypertrophy during early muscle growth can be differentially affected by environmental temperature. Vieira & Johnston (1992) found that 1-day old herring larvae reared at 15°C had greater numbers of muscle fibres, but that the mean cross-sectional area of these fibres was lower, so that the total cross-sectional area of muscle was unaffected by temperature. This suggests that rearing at a high temperature favoured fibre recruitment relative to hypertrophy. Subsequent studies have indicated a considerable degree of interannual variation in the effects of temperature on muscle cellularity in herring (Johnston 1993; Johnston *et al.* 1995). Stickland *et al.* (1988), studying Atlantic salmon embryos, found the reverse situation to that found in herring by Vieira & Johnston (1992) - in salmon, high rearing temperatures resulted in larger but fewer fibres, implying that recruitment had been relatively reduced but that hypertrophy had been increased to compensate. Hanel *et al.* (1996), studying the whitefish (*Coregonus lavaretus* L.), and Thomas (1998), studying the shorthorn sculpin (*Myoxocephalus scorpius* L.), also described a shift towards reduced recruitment and greater hypertrophy at high temperatures.

The thermal regime experienced by the embryo can also affect muscle development in ways that can have consequences for muscle growth later in life. Spring-spawned herring embryos reared at 8°C have greater numbers of myosatellite cells upon hatching than embryos reared at 5°C (Johnston 1993), and subsequently grow more quickly than the 5°C-reared fish even when both groups are transferred to a common temperature after first feeding (Johnston *et al.* 1998).

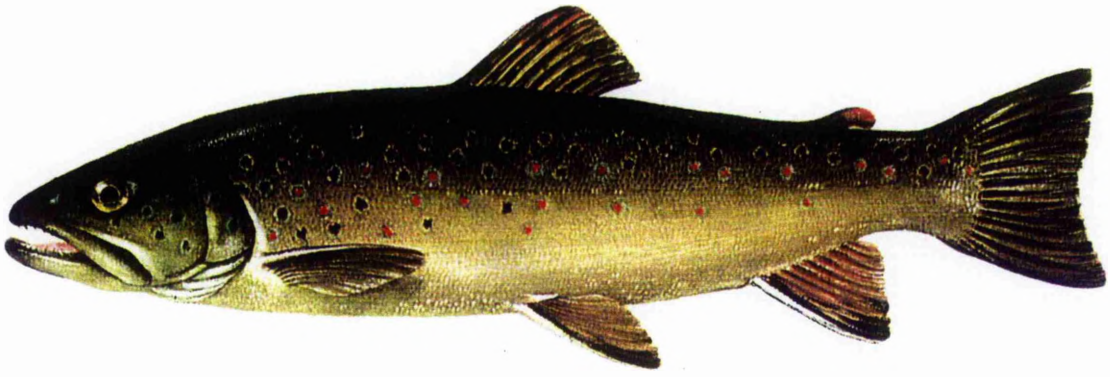


**Fig. 1.12.** The brown trout, *Salmo trutta*. From Migdalski & Fechter (1976).

## The brown trout, *Salmo trutta*

### *The distribution of Salmo trutta*

The brown trout (Fig. 1.12), is a native of Europe, and is widespread as far south as the Mediterranean coast and the Atlas mountains in North Africa, and as far east as the Ural mountains and the Caspian Sea (Fig. 1.13). In 1852, the first artificial introduction of brown trout outside Europe took place in Eastern Russia, and since then the species has been successfully introduced to at least 24 countries (Fig. 1.13). The potential range of the species is probably defined by its water temperature requirements - 0 - 15°C for successful egg development, and 4 - 19.5°C for growth in juveniles and adults (Elliott 1994). Optimum growth is at temperatures above 15°C (Jensen & Berg 1995). Like all salmonids, trout also have high oxygen requirements, preferably 80% of saturation (Mills 1971, cited by Elliott 1994). Other important criteria include availability of appropriate spawning grounds, water quality and flow rates, food supply and predation (Heggenes & Traaen 1988).

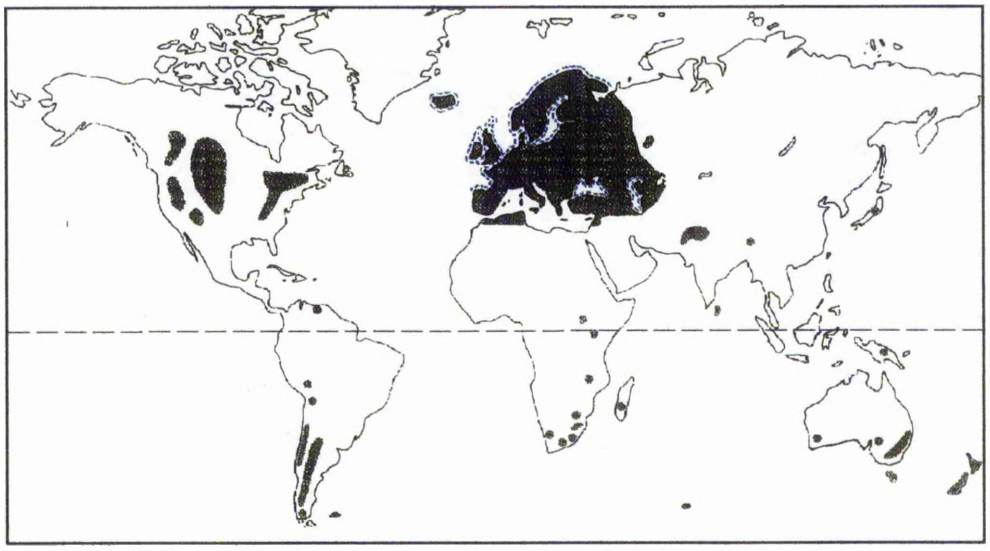


**Fig. 1.13.** World map illustrating the distribution of *Salmo trutta*. The European distribution of anadromous *S. trutta* is illustrated by a dashed blue line. After Elliott (1994).

The brown trout is a member of the Family Salmonidae. The family is divided into two subfamilies, the Thymallinae (graylings) and the Salmoninae (charr, salmon and trout) (Sanford 1990). All salmonids spawn in fresh water, where they are believed to have originated, and can be distinguished by the presence of the adipose fin, a small, fleshy fin between the dorsal and caudal fins. The species currently recognised as the brown trout, *S. trutta*, was first classified as such by Regan (1911, cited by Elliott 1994), although over the years, different varieties of brown trout have been separately assigned to over fifty distinct species by assorted authors (Behnke 1986). Even today, some workers still divide *S. trutta* into two separate subspecies, *S. trutta trutta* and *S. trutta fario*. The main source of this confusion is the broad range of life histories present in the species, which reflects a general trend for high variability in life history strategy in the family Salmonidae.

***Life history variation in S. trutta***

Like other salmonids, brown trout usually bury their eggs in nests, or 'redds', constructed during spawning in the gravel beds of rivers and tributaries. In the Northern hemisphere, spawning most commonly occurs in November and December; the embryos develop over the winter, and after hatching remain in the gravel, utilising their remaining yolk supply. When this is nearly exhausted (Jensen *et al.* 1989), the young fish emerge into the stream and begin feeding. Although there is some degree of confusion over the terminology of early salmonid life stages, the term 'alevin' is usually used in reference to the free embryos, between hatching and emergence. Fish in the brief transition period from endogenous to exclusively exogenous feeding are often called 'fry'. 'Parr' refers to the period of feeding and growth in the stream from the time of yolk exhaustion onwards (young parr have distinctive vertical patches of pigment called 'parr marks').

After the first year of life in fresh water, the variability in the life history begins to manifest itself. Trout which follow the simplest life cycle never leave their natal stream; they typically mature after two to three years, and may spawn several times before death. Other trout migrate for part of their life cycle to the main stem of the parent river or a lake before returning to the stream where they were hatched. Some fish take this migratory tendency further, and spend part of their lives in an estuary or at sea, where growth rates are improved (Elliott 1994). Migration to salt water is preceded by a suite of physiological and morphological changes, known as smoltification. Anadromous trout usually return to their home river each winter, returning to the sea in the spring to feed (Nordeng 1977). If they are mature, the trout travel upstream to their natal tributary, mate, and spawn. Trout are iteroparous, being capable of spawning more than once. The term 'brown trout' is often used in reference to freshwater resident fish only, while anadromous *S. trutta* are called 'sea trout'.

The range of migratory habits, from full freshwater residency to anadromy, also exists interspecifically across the family Salmonidae - certain Pacific trout species such as *Oncorhynchus apache* Walbaum and *O. gilae* Walbaum never leave fresh water, while pink salmon (*O. gorbuscha* Walbaum) always migrate to sea, almost directly after emergence. While Atlantic salmon are usually anadromous,

there are some populations that are fully fresh water resident (Berg 1985). Partial migration, the phenomenon of populations made up of migratory and non-migratory individuals, has been documented in many animal taxa, from insects to higher vertebrates (reviewed in Jonsson & Jonsson 1993).

The species *S. trutta* is naturally substructured into populations that are largely reproductively isolated and genetically distinct, within as well as among drainages; the genetic differentiation between populations is maintained by homing (LeCren 1985). Populations can contain a sympatric mix of different migratory types (Allendorf *et al.* 1976), the proportion of each type varying markedly between populations (Jonsson 1982). Anadromous adults can be distinguished from freshwater residents on the basis of scale growth patterns - the faster growth that occurs at sea results in a more open pattern of circuli (Frost & Brown 1967). Alternatively, migratory types can be distinguished on the basis of the carotenoid pigment content of the tissues (Youngson *et al.* 1997). Carotenoids, which impart an orange-to-red colour to the flesh, are assimilated by salmonids from their diet, which will differ according to the life history. The tissues of anadromous trout contain relatively more astaxanthin, a marine pigment, while resident fish have more lutein (Youngson *et al.* 1997). The carotenoid profile of the female parent is reflected in the eggs, and can persist in the offspring as late as the fry period (Craik & Harvey 1986).

***Genetic factors affecting the migratory type of a trout***

Freshwater residency is more frequent among male trout, up to 90% (Jonsson 1985), which often mature at a small size as 'precocious' parr (Campbell 1977). Sex-ratios of anadromous fish are often skewed towards a greater proportion of females (Jonsson 1985), suggesting a sex-linked genetic component. Anadromy may be more common in females because growth rates at sea are much greater than those in fresh water, and female fecundity and overall reproductive fitness are strongly linked to body size (Thorpe *et al.* 1984). Van den Berghe & Gross (1989) reported that increased body size could confer a 23-fold fitness advantage to the largest females of Pacific salmon (*Oncorhynchus kisutch* Walbaum).

There have been reports of small freshwater resident males fertilising the eggs of larger anadromous females, suggesting a lack of assortative mating (Campbell 1977). Although anadromous males have not been seen to fertilise the eggs of small resident females, this may be due to the relative scarcity of these types (Thompson 1995). Frost & Brown (1967) found that resident trout could produce anadromous offspring, while offspring of anadromous fish can follow a resident life history (Rounsefell 1958, cited by Jonsson & Jonsson 1993). However, resident parents do produce relatively more resident offspring than anadromous parents (Skrochowska 1969). Between-stock differences in migratory tendency are maintained when trout from different stocks are released together (Svärdson & Fagerström 1982, cited by Jonsson 1985), implying that migratory type is at least partially genetically determined.

Like *S. trutta*, the Pacific salmon (*Oncorhynchus nerka* Walbaum) also has both anadromous (sockeye) and resident (kokanee) morphs, sympatric populations of which differ significantly in their minisatellite DNA allele frequencies (Wood & Foote 1990). Attempts to find a similar genetic basis for determination of migratory type in sympatrically co-existing trout have produced mixed results. Hindar *et al.* (1991), studying isozyme variation in the Voss river system in western Norway, found that differences between migratory types within spawning sites accounted for only 0.2% of the total variation, compared to 1.7% arising from differences between spawning sites. Cross *et al.* (1992) also found no significant allele frequency differences between migratory types in the Burrishoole river system, although slightly higher levels of heterozygosity were evident in the anadromous fish. In contrast, Makhrov *et al.* (1997) reported higher heterozygosity in stream-resident males. Thompson (1995) found no significant differences in heterozygosity between migratory types, but her studies on mitochondrial DNA variation produced some evidence of a heritable basis for migratory type, connected with the maternal phenotype. Similar findings have been reported for chinook salmon (*Oncorhynchus tshawytscha* Walbaum) (Clarke *et al.* 1994).

This maternal influence on migratory type may be genetic in nature, or may involve differences in choice of spawning sites, depth of egg burial, or egg carotenoid pigment content (Thompson 1995). Egg size may also differ between

migratory types. In a comparison between migratory female trout from the Black Brows Beck and landlocked resident females from the Wilfin Beck, both in the Windermere catchment and separated by just three kilometres, the migratory females were larger, produced more and larger eggs, and overall invested approximately twice as much into egg production, in terms of the female's total energy content (Elliott 1994).

***The environment and migratory type***

Such maternal influences, be they genetic or otherwise, may alter the growth rate of the offspring. Inherited factors may also "modify the response to environmental cues in different populations" (Thompson 1995). Jonsson (1985) considered that "both genetics and environment influence whether salmonids become residents or migrants". In Arctic charr (*Salvelinus alpinus* L.), the proportion of residents versus anadromous fish varies with feeding intensity (Nordeng 1983); improved juvenile feeding tends to increase the proportion of resident fish. Temperature could affect salmonid life history in a similar way to feeding intensity, as both variables affect fish growth (Jonsson 1985).

An increase in latitude is associated with reductions in temperature and in the length of the growing season (normally defined as the number of days where temperature exceeds 4°C). Although there is some evidence that salmonids exhibit a degree of latitudinal compensation (Conover 1990), annual growth rates at sea and, more importantly, during the freshwater parr period, are still lower at higher latitudes, which leads to higher mean smolting ages in many anadromous salmonid species (Parker & Larkin 1959). It is important to note, however, that the age at which smolting occurs is not dependent solely on the attainment of a threshold size - smolt size increases with smolting age. Due to their lower energy requirements, slow-growing fish may not need to migrate to the improved feeding opportunities at sea as early as fast-growing fish (Økland *et al.* 1993). Interestingly, in their European distribution (Fig. 1.13), resident trout are found as far south as approximately 35°N, but anadromous trout are only found at latitudes greater than 42°N (Elliott 1994) - implying a connection between anadromy and higher latitudes, and therefore lower temperatures and growth rates.

It is also possible that the migratory type of the adult may be connected to, or reflected in, developmental changes as early as the embryonic and free embryo periods. As discussed earlier, the relative timings of such changes may be affected by environmental variables such as temperature. Balon (1990) theorised that "such epigenetically introduced changes... (may) ...lead to alternative life histories even in a clutch from the same parent". Although Gorodilov (1989) found no differences in the early ontogeny of different migratory forms of *S. trutta*, at a range of temperatures, differences have been reported between migratory types in the somite stages at which structures including otic vesicles and the lenses in the eyes were formed (Halacka 1995).

## The present study

All fish used in this study were progeny of wild trout (both anadromous and freshwater resident), reared in a hatchery under a variety of temperature regimes. The results of the investigations carried out for this thesis are presented in Chapters 2 to 6.

Chapter 2 contains a detailed description of early trout ontogeny, and details new methods for quantifying the degree of development of individual embryos. Shifts in the relative timing of certain aspects of development with temperature are also described.

In Chapter 3, the relationship between growth (i.e. increase in size) and development (i.e. differentiation) is examined in trout embryos and alevins reared at different temperatures, in conjunction with studies on variables such as egg size and the efficiency of yolk utilisation.

In Chapter 4, particular attention is paid to the effects of temperature on development of the first trunk muscle tissue. The timing of formation of the first myotubes and muscle fibres is described, based on examination of histological specimens, and certain aspects of early neural development are also examined.

Chapter 5 describes the patterns of muscle growth during the embryonic and alevin periods in trout reared at two different temperatures, 10°C and 2°C. Changes in the muscle cellularity are analysed and examined relative to both the degree of development and the total size of the fish.

In the final results chapter, Chapter 6, the possibility that the temperature experienced by a fish during its earliest life stages can affect later potential for growth is investigated. Growth potential is examined in the context of the ability of fish to improve their rate of growth in response to increased levels of swimming exercise.

The main findings of this thesis are discussed, and potential future areas of research suggested, in the General Discussion (Chapter 7).

# Chapter 2: A quantitative scoring method used to determine the effects of temperature and maternal migratory type on development in the trout (*Salmo trutta*)

## Introduction

Temperature has long been known to have significant effects on the development of teleost embryos. Most obviously, rearing in warmer water results in accelerated growth and development. Temperature during embryonic development can also affect the phenotype of the adult, modulating meristic characters such as vertebral and fin ray numbers (Seymour 1959; Beacham & Murray 1986), and may alter the relative timing of certain developmental changes. Various studies on salmonid embryogenesis have reported temperature-induced variations in the timing, relative to overall developmental state, of epiboly, first heartbeat, pelvic fin formation, skeleton and digestive system formation, aspects of pigmentation, and hatching (Hayes *et al.* 1953; Ballard 1973c; Balon 1980; Pavlov 1984; Rombough 1987). Heterochrony during embryonic development has been suggested as a mechanism of evolutionary change (Freeman 1982). However, Vernier (1969) found no effect of temperature on the relative timing of formation of anatomical structures in rainbow trout (*Oncorhynchus mykiss*); only physiological changes, such as first production of bile in the intestine, varied in their timing relative to the rest of embryogenesis. Similar findings were published by Garside (1959) and Gorodilov (1983; 1989; 1996).

It is possible that temperature during early development may also interact with genotype to affect migratory habit in salmonid species such as the trout (*Salmo trutta*) (Jonsson 1985). Factors determining the migratory phenotype are little understood. Allele frequency studies have found no significant differences between coexisting migratory and non-migratory forms (Hindar *et al.* 1991). However, Thompson (1995) found that mitochondrial haplotype frequencies differed significantly between migratory groups within a population, suggesting a maternal effect on the migratory type of offspring. As the anadromous morph is more prevalent in streams at higher

latitudes, with lower ambient temperatures (Elliott 1994), temperature-dependent heterochrony during embryogenesis may interact with the migratory type of the female parent to determine that of the offspring. Gorodilov (1989) compared the embryogenesis of three forms of *S. trutta*, at 0.6, 6.2 and 12.2°C, and found no effects of temperature or maternal migratory type on developmental patterns. Halacka (1995) found that several aspects of development, such as the formation of otic vesicles, occurred at a later somite stage in freshwater resident trout reared at 1.6 - 1.7°C, relative to offspring of migratory sea trout reared at 4.8°C (Pavlov 1989).

The work by Halacka (1995) constitutes the most recent description of embryonic development of *S. trutta*, but covers only the pre-hatch period, which is classified into just nine subdivisions. This limits its precision as a tool for describing variations in the rate of development between individual embryos. Also, the rearing temperatures used in this study were not constant, but varied from 0.5 to 5°C. Gorodilov (1989) studied the embryogenesis of *S. trutta* alongside that of Atlantic salmon (*S. salar*) over a range of constant temperatures, and found the developmental patterns to be identical. His most recent description of salmon development (Gorodilov 1996) is detailed and extends to the onset of exogenous feeding. It subdivides embryogenesis into a series of successive 'states'; however, each state, or interval of development, is defined on the basis of a single meristic character (e.g. the 10-somite state; the 14-caudal fin ray state), even though each state will encompass a variety of other developmental changes. This system assumes that all developmental patterns are absolutely canalised,i.e. that the occurrence of one change automatically implies the occurrence of all associated changes for that state. This is not always true even under uniform conditions (Armstrong & Child 1965). Also, as only some of these states are described, separated by gaps of irregular duration, the work can in places resemble a more traditional 'staging series' i.e. a list describing embryos at a number of single points in ontogeny, rather than a more fluid description of continuous change.

The primary aim of the study described in this chapter was to determine the effects of temperature, and of maternal migratory type, on the early development of *S. trutta*. To facilitate this, and also to expand on previous studies of early salmonid

ontogeny, a detailed description of trout development was prepared. This is presented below as a series of forty developmental 'steps'; like states, each step covers a certain subdivision of the total embryonic period, from fertilisation of the egg to the time when the fish were ready to make the transition to exogenous feeding ('first feeding'). However, the steps describe a number of changes occurring during this subdivision, and classification is therefore based on assessment of all features of a step, not just one. For even more precise assessment of the level of developmental advancement, a quantitative 'scoring system' was devised, in which individual embryos are assigned points on the basis of separate assessment of a wide range of developmental changes, or 'features'. In addition, the use of certain meristic features as indicators of developmental advancement in the period immediately after first feeding was investigated.

## Materials and Methods

### *Embryo maintenance and sampling*

Fish used in the experiment, supplied by the Don District Salmon Fishery Board, Scotland, were progeny of mature male and female trout, captured in advance of spawning in the upper reaches of the River Don. Gametes were stripped and transported on ice to the Fisheries Research Services Salmon Rearing Unit, Almondbank, Perthshire, Scotland. Development was studied in two consecutive years, 1995 and 1996. In October of each year, equal numbers of eggs from each of ten females were fertilised with milt from six males. After being covered in water and allowed to 'water harden' for approximately one hour, the eggs were recombined to produce ten maternal groups, each with a mix of male parents. In 1996, a second fertilisation was carried out two weeks after the main series, in which eggs from a single anadromous female were fertilised with milt from two males to produce a single maternal group. The sizes of the parental fish used are listed in Appendix I. The migratory type of the female parent was identified on the basis of the carotenoid pigment content of unfertilised eggs (5 - 10 eggs per female), which is a function of maternal dietary history. Pigment contents were determined using isocratic normal phase HPLC analysis, after Noack *et al.* (1996) (See Appendix II).

In 1995, fertilised eggs from each maternal group were subdivided among egg trays (11 x 11 x 15cm) with perforated bottoms, suspended in tanks supplied with river water at the rate of 4 $l.min^{-1}$. The water flowed under the trays and entered through the perforations, passing up through the layer of eggs before exiting the tray at the top and continuing through the tank. Water was maintained at three controlled temperatures - 10°C, 6°C and 2°C. Typical temperature ranges measured over a period of 2 months were 9.8-10.4°C, 5.6-6.2°C and 1.6-2.4°C; sample temperature records are presented in Appendix III. In 1996, the eggs from the first fertilisation were reared under the 10°C or 2°C regimes; the eggs from the second fertilisation in 1996 were all maintained under the 6°C regime. Egg trays were arranged in rectangular tanks in a 4 x 5 array; at each temperature, the eggs from each maternal group were split among two egg trays, arranged to minimise net between-group differences in position within the tank.

Embryos (n=6) were sampled regularly from all groups. For embryos from the two main fertilisations, the initial sampling rate was daily at 10°C, every two days at 6°C, and every four days at 2°C. Embryos from the second fertilisation, in 1996, were initially sampled every four hours until the 32-cell stage, then daily. The sampling rate was reduced as the embryos developed and the rate of ontogenetic change slowed, so that just prior to the stage at which the free embryos were ready to begin feeding, samples were being collected every 4-5 days at 6°C. Eggs were examined in Petri dishes of distilled water under a Zeiss Stemi SV 6 stereo dissecting microscope, at magnifications between 8x and 50x. Between the levels of approximately 50% epiboly and hatching, the eggs were transferred to phosphate buffered saline (PBS) and the embryos were removed from the chorion with the aid of sharpened No. 5 watchmakers' forceps for further examination. Once embryos began to display spontaneous movement, they were anaesthetised after initial examination by addition of drops of 0.02% m/v MS-222 (ethyl m-aminobenzoate). After examination, the embryos were fixed in buffered 10% formalin overnight, then transferred to 0.1% sodium azide in PBS; additional observations were made on these fixed specimens.

***Development after first feeding***

Fish from the second fertilisation, reared at 6°C, were transferred after first feeding to 1m diameter tanks with a water flow rate of 5 $l.min^{-1}$. Water temperature was maintained at 13°C; the photoperiod was 16L:8D. Fish were supplied with salmon starter food (Ewos, Bathgate) at a rate of approximately 5% body $mass.day^{-1}$. Samples of 6 - 10 fish were collected every four days initially, decreasing to approximately every 10 days by 80 days after first feeding, when sampling ceased. Counts were made of the following features: branchiostegal rays, dorsal and ventral procurrent caudal fin rays, rows of segments at the caudal fin rays and rows of segments at the anal fin rays

***Preparation of steps and the scoring system***

Observations made at all three temperatures, from both years, were used to construct the final descriptions used in the list of steps. For the scoring system, however, the exact timings, and thus the point values, for each feature were based solely on the embryos from the second fertilisation of 1996.

Development from fertilisation to first feeding was divided into a sequence of 40 'steps', each step describing the changes occurring over a certain interval of development. The boundaries between steps were set at points where distinct and easily visible developmental changes occurred. The duration of succesive steps gradually increases through the sequence, to reflect the manner in which the intensity of developmental change is known to decrease with age (Balon 1975b).

The steps were also grouped together into six 'phases': cleavage egg (C), gastrula (G), somitogenesis (S), yolksac vascularisation (Y), caudal fin ray formation (F) and finfold resorption (R). (Note: The names for the phases are chosen for convenience; the boundaries between the phases do not necessarily represent the beginning and end points of the actual processes. For example, true cleavage ends before the end of the 'cleavage egg' phase, while somite formation continues past the end of the 'somitogenesis phase'.) Each step could thus be named according to its position in the entire sequence (e.g. Step 12) or according to its position in the

relevant phase (e.g. Step G3 - the third step in the gastrula phase.) Steps were also named according to the feature that normally marks the beginning of that step (e.g. Neural Plate Formation step), but this should not be used as the sole diagnostic feature for that step.

For the construction of the scoring system, a total of 1000 points were assigned to the period from fertilisation to the estimated time of first feeding - 127 days at 6°C. Thus each day of development corresponded to approximately 7.9 points. A list of 164 developmental features was compiled, avoiding any whose relative timing was found to vary substantially with temperature. Wherever possible, the features used involve definite, discrete transitions (e.g. angles of fin outlines), in order to minimise subjective error. In addition, five different meristic features were chosen, counts of which contribute to the final score during certain phases of development. These were somite pairs, caudal fin rays, segments in the caudal fin rays, branchiostegal rays, and ventral procurrent caudal fin rays (V.P.C. fin rays). Point values were assigned to individual features in such a way that, as a trout embryo develops and successive features appear, the sum of the point values for the features will increase in a straight line relative to age. The scoring system is thus designed to be used as a tool, to determine the age of embryos based on visual inspection.

The points assigned to an individual feature depend on the length of time taken for it to become present in all embryos in a sample, and also on the number of other features used over the same time period. Thus, the features "Circulation at midbrain-hindbrain boundary", "Ventral mesenchyme thickens at future caudal fin", "Mesenchyme dorsal to somites" and "Head free from yolk as far back as upper jaw" are each worth 2 points, as all four develop over the same single-day period during Step 21 (Y1), worth a total of 7.9 points. A small degree of rounding-off of figures was necessary to avoid use of decimal points wherever possible.

As a later example, the features "Pelvic fins wider at middle than at bases", ">6 dorsal fin rays" and ">3 anal fin rays" become present in all embryos over a three-day period, worth a total of 23.7 points, during Step 33 (F7). Of this, 6.9 points are assigned to the increase in caudal fin ray number over the same period (three days,

0.46 rays formed per day, 5 points each). So the remaining 16.8 points are divided among the three features listed above - 5 points for "Pelvic fins wider...", 6 points each for ">6 dorsal..." and ">3 anal...".

To enable more rapid calculation of the score, nineteen features were selected to be used as 'landmark features', each with an associated 'base level score' as well as the normal point value. Where possible, the landmark features correspond to changes that herald a transition from one step to another. The purpose of these landmark features is to guide the user to the most relevant part of the scoring system before the final score is calculated. A second version of the scoring system was produced for use with study of fixed specimens. This version does not rely on any circulatory features, which cannot be discerned after fixation. Proportions and angles of fins, and the posterior extent of the operculum, have also been excluded, as these can change differentially with fixation. Because of this, the post-fixation scoring system must necessarily be less precise than the version for live specimens. Also, in some cases different landmark features are used.

***Effects of temperature and maternal migratory type on development***

Features whose timing of appearance differed substantially with temperature, relative to the rest of development, were noted in both years. The developmental scoring system was prepared based solely on observations of embryos of the maternal group from the second fertilisation in 1996, reared at 6°C; however, values of developmental score were subsequently determined for embryos reared at 10°C and 2°C, from a mix of maternal groups in 1996. This was to determine if the linear nature of the increase in score with age was maintained at temperatures other than 6°C. The effects of maternal migratory type on the relative timings of developmental changes could only be studied effectively in the 1995 fish, when the ten females obtained consisted of four anadromous and six freshwater resident fish; in 1996, only two of the ten females obtained proved to be anadromous.

Rates of somite formation for offspring of anadromous and of resident females were compared by GLM ANCOVA (General Linear Model Analysis of Covariance) using the Minitab statistical analysis package (Minitab Inc., USA); temperature and

maternal migratory type were examined as factors, and age (expressed as days post-fertilisation) was used as a covariate. The final number of somites was determined in 20 embryos of each migratory type, at each temperature, from a mix of maternal groups in 1995. Counts were only taken from embryos at steps 21 (Y1) - 23 (Y3), as the number of somites clearly visible is reduced from step 24 (Y4) onwards (Gorodilov 1996). The results were analysed by ANOVA (Analysis of variance), using temperature and maternal migratory type as factors. The procedure was then repeated using temperature as the sole factor, followed by Tukey multiple comparison tests.

## Results

### *Developmental steps*

A series of 40 developmental steps is given below, along with the normal time span of each step at 6°C, and the typical range of point scores occurring within that step. Each step has a particular feature that normally heralds the transition to that step from the previous one; but an embryo may be assigned to a certain step even if the 'heralding' feature is absent, if the embryo bears a number of other features from that step. This is not an uncommon situation given the natural low level of heterochrony of development even under identical conditions.

Step 0 - Unfertilised egg (Fig. 2.1, p. 84)

The unfertilised, unactivated egg is not spherical, being quite irregular in shape, with numerous relatively flat surfaces. Large numbers of small oil droplets are scattered randomly over the egg surface, lying just underneath the egg capsule or chorion, which fits tightly around the viscous yolk but is still soft and flexible.

*Cleavage egg phase (Fig. 2.1, p. 84):*

The term 'cleavage egg' is used to describe the embryo from the time of fertilisation up until the formation of the germ ring; it includes the blastula phase often defined by other authors (e.g. Kimmel *et al.* 1995; Gorodilov 1996), as the end of synchronous cleavage is difficult to distinguish. Time intervals given for the initial cell divisions are approximations only.

Step 1 (C1) - 1 Cell (Fig. 2.1)

0H - 11 hours (1 - 3 points)

After activation and fertilisation, the egg swells and becomes rounder, although it is still not perfectly spherical. Within an hour, the chorion has become hardened, and a large fluid-filled perivitelline space has formed at the top of the egg. The surface of the yolk beneath this space is flattened, and is partially covered by a disc of concentrated oil droplets, approximately half the diameter of the egg. Cytoplasm begins to aggregate above this disc of droplets, so that by 3 hours post-fertilisation, a flattened, convex blastodisc is visible at the animal pole when the egg is viewed from the side. By the end of this step the blastodisc has increased in size, so that its diameter is approximately 1/6 - 1/4 that of the egg, and it is roughly 1/3 tall as it is wide. The edges of the blastodisc are slightly undercut from the underlying yolk. When viewed from above, a pale 'halo' is visible between the blastodisc and a surrounding elevated ring of large oil droplets.

Step 2 (C2) - 2 Cell (Fig. 2.1)

11 - 18.5 hours (5.5 points)

The blastodisc divides by meroblastic cleavage, in a plane perpendicular to the base (i.e. meridional). The resulting blastomeres appear similar, but not always identical, in size. The blastodisc is now longer in the plane perpendicular to the first cleavage plane.

Step 3 (C3) - 4 Cell (Fig. 2.1)

18.5 - 26 hours (8 points)

The embryo continues to divide, with a cell cycle time of approximately 7.5 hours at 6°C. The second cleavage furrow is also meridional, and develops perpendicular to the first. When viewed from above, the blastodisc has the appearance of a rounded square.

Step 4 (C4) - 8 Cell (Fig. 2.1)

26 - 33 hours (10.5 points)

Variations in the pattern of cleavage appear during this step. In some embryos, two meridional cleavage furrows develop parallel to the first, resulting in a 4 x 2 arrangement of blastomeres in a blastodisc that is substantially longer in one axis. In others, however, the two new furrows are perpendicular to each other, and at 45° to the earlier cleavage planes, producing a rosette-shaped blastodisc, as described by Battle (1944). Inequalities in the size of the cells become increasingly obvious.

Step 5 (C5) - 16 Cell (Fig. 2.1)

33 - 40.5 hours (13 points)

The fourth cleavage is again meridional, although the planes of the individual divisions can no longer be clearly distinguished. The blastodisc is still one cell thick when viewed from the side, although because of its concave-convex shape, the central blastomeres appear taller than the peripheral ones.

Step 6 (C6) - Mid-Cleavage (Fig. 2.1)

40.5 hours - 3 days (23 points)

The fifth cleavage is in the horizontal plane, parallel to the base of the blastodisc. From this point onwards, the number of cells becomes increasingly difficult to count accurately. The blastomeres continue to divide without any increase in the overall size of the blastodisc. In side view, the blastodisc appears approximately 3 - 9 cells tall (not visible through the chorion), although the irregular arrangement of the blastomeres means that the exact number of stacked cells is a poor indicator of the state of development.

Step 7 (C7) - Late Cleavage (Fig. 2.1)

3 - 5 days (39 points)

The dividing cells become so small as to be barely discernible under a dissecting microscope. The blastodisc is approximately 10 - 15 cells tall in side view,

but does still not appear any larger than when at the 1-cell stage. At the end of this step, the edges of the embryo are no longer undercut, but begin to slope outward to a greater extent where they contact the yolk.

Step 8 (C8) - Early Blastula (Fig. 2.1)

5 - 7 days (47 - 55 points)

Individual cells can no longer be distinguished under a dissecting microscope. The 'halo' that surrounded the blastodisc when viewed from above has disappeared, as the embryo begins to flatten and the edge spreads outward. The periphery of the disc of oil droplets beneath the blastodisc is no longer elevated.

Step 9 (C9) - Late Blastula (Fig. 2.1)

7 - 9 days (71 points)

The lower edges of the flattening blastodisc no longer meet the yolk at an angle; instead the gently sloping sides of the convex blastodisc are contiguous with the edge of the yolk.

*Gastrula phase (Fig. 2.1, p. 84):*

The term 'gastrulation' is used to describe a combination of three processes: convergent extension, epiboly, and involution at the germ ring.

Step 10 (G1) - Germ Ring Formation (Fig. 2.1)

9 - 11 days (87 points)

The edge of the blastodisc now consists of a distinctly thickened rim - the germ ring. When viewed from the side, this may be visible as 'shoulders' on either side of the otherwise smooth blastodisc. The diameter of the blastodisc, which has been stable at approximately half that of the egg, begins to increase once more as epiboly begins.

Step 11 (G2) - Embryonic Shield Formation (Fig. 2.1)

11 - 13 days (95 - 103 points)

As epiboly progresses and the blastodisc thins, the imprint of the original size of the blastodisc is visible as a darker circle at the centre. Cells begin to accumulate at the germ ring on one side of the blastodisc, forming a thickened node, the embryonic shield. By the end of this step, this consists of a triangular-shaped wedge of cells - the wide base lies at the outer edge of the germ ring, while the blunt point of the triangle protrudes inwards past the periphery of the 'imprint'.

Step 12 (G3) - Neural Plate Formation (Fig. 2.1)

13 - 15 days (107 - 111 points)

At the beginning of this step, tissue becomes especially concentrated along the median vertical axis of the embryonic shield, starting at the base and progressing towards the top (anterior). This is the early neural plate. The embryonic shield continues to increase in size - as epiboly progresses, its base moves towards the equator of the egg, and its upper portion, which will form the head of the embryo, extends towards the animal pole. In smaller eggs, the germ ring reaches the equator.

By the end of step G3, the upper, cephalic portion of the gastrula has become slightly wider than the more posterior portion. This is visible through the chorion. If the embryo is removed from the yolk, a wedge of cells is seen protruding ventrally; anteriorly, it is substantially thicker than the part of the embryo that lies above the yolk. This is the neural keel, formed by the infolding of the neural plate, and in an intact embryo it projects into the surface of the yolk.

It is during the later part of this step that the first two to four pairs of somites are formed from the axial mesoderm on either side of the developing neural tissue, approximately two-thirds of the distance from the head to the posterior extremity. However, these are extremely difficult to distinguish, and it would be unreliable to use them as a diagnostic feature for this stage.

*Somitogenesis phase (Fig. 2.2, p. 85):*

The distinction between the gastrula and somitogenesis phases is relatively artificial, based on when the somites become visible rather than when they are calculated to have first been formed. As gastrulation is considered to include epiboly, it can persist as late as step 16 (S4).

At 6°C, somites are formed at the rate of 3.74 somites.day$^{-1}$ (based on the 1996 fertilisation; the rate of somite formation was slightly lower in the 1995 fertilisation - Fig. 2.9, Table 2.5).

Step 13 (S1) - Early Brain Differentiation (Fig. 2.2)

15 - 17 days (119 - 131 points)

4 - 10 somites

At the beginning of S1, the anterior portion of the neural keel, already wider (when viewed dorsally) than the more posterior portion, begins to differentiate into the fore-, mid- and hind-brain. At the same time, the optic primordia become visible as large buds of tissue on either side of the forebrain. A median groove can be seen in the neural keel, which still indents the yolk for most of the length of the embryo. This indicates the onset of primary neurulation.

In smaller eggs, the head reaches the animal pole. In side view, the blastoderm may have progressed as much as three-quarters of the way to the vegetal pole. Epiboly progresses more slowly at the site of the embryo than elsewhere along the germ ring.

Step 14 (S2) - Otic Placodes (Fig. 2.2)

17 - 19 days (131 - 147 points)

10 - 18 somites

The otic placodes become discernible as tiny ovals of tissue lateral to the hindbrain at the beginning of S2. By the end of this step these placodes have swollen

and begun to develop lumina as they transform into the otic vesicles. The neural keel becomes the neural rod, and thins until it no longer indents the yolk.

If the embryo is removed from the yolk, the notochord can be seen running ventrally to the neural rod, extending from behind the otic placodes to the position of the most posterior somite. Numerous closely-packed vertical ridges give it the 'stack-of-pennies' appearance described by Kimmel *et al.* (1995).

Kupffer's vesicle can be seen in sectioned embryos, although not in intact specimens as it is obscured by the assorted oil globules attached to the ventral surface. This small, fluid-filled cavity with a dorsal lining of columnar epithelium is located ventrally within the tailbud, and persists until steps S4 - S5, when the tailbud frees from the yolk. Some authors (e.g. Armstrong & Child 1965; Iwamatsu 1994) have described Kupffer's vesicle as consisting of oil globules or vacuoles located in the yolk, ventral to the tailbud, but in *S. trutta* it is enclosed within the body of the embryo, as in zebrafish (Kimmel *et al.* 1995).

When embryos at the end of S2 are viewed dorsally, regions of thickened tissue directly overlying the yolk may be visible lateral to the anterior portion of the hindbrain, and lateral to the anterior-most somites. These represent the sites of formation of the future pericardial cavity and the pronephri (kidney rudiments) respectively.

By the end of S2, epiboly is completed in the smallest eggs, but in most a region of exposed yolk (the blastopore or yolk plug) is still present, located posteriorly to the end of the tailbud.

Step 15 (S3) - Optic Cup Formation (Fig. 2.2)
19 - 21 days (147 - 163 points)
18 - 26 somites

S3 begins with the development of a groove in the anterior-dorsal region of each optic placode. By the end of the step this groove has extended to form a

complete oval concentric to the edge of the placode, delineating the area of ectoderm that will form the lens. The rest of the optic placode forms the optic cup, which will develop into the retina.

The brain continues to differentiate; part of the forebrain swells to form the ventrally-protruding diencephalon, while all but the anterior-most part of the hindbrain segregates into 5 distinct neuromeres.

The otic vesicles contain complete lumina by the end of S3. In the region of the hindbrain, the surface of the yolk sinks away from the embryo, forming the pericardial cavity, although it is still small compared to its later size. The heart rudiments are not yet easily visible. Ventral to somites 3-5, the paired pronephri form, although these are also difficult to distinguish unless the yolksac is removed. The pronephric ducts are still undifferentiated.

Epiboly is completed in most, but not all eggs.

Step 16 (S4) - Chevron-shaped Somites (Fig. 2.2)
21- 22 days (159 - 175 points)
26 - 32 somites

Step S4 begins with the onset of the change of shape of somites 4 - 10 (approximately) from simple rounded rectangles to chevrons pointing anteriorly (in side view).

The midbrain and the most anterior part of the hindbrain (the cerebellum) are now taller than the rest of the hindbrain, which is separated from the dorsal epithelium by a fluid-filled cavity. A vertical constriction develops at the midbrain-hindbrain boundary, while at the same time a horizontal groove appears in the midbrain, just behind the tops of the eyes. The latter subdivides the midbrain into the dorsal optic tectum and the ventral midbrain tegmentum. The choroid fissure, a gap in the optic cup, becomes defined at the bottom of each eye.

The pericardial chamber increases in size throughout S4, so that by the end of the step the heart is clearly visible as a vertical cone. The broad base sits on the yolk surface, while the apex joins the embryo just anterior to the position of the otic vesicle. In the most advanced embryos the edges of the cone are slightly indented in the middle, indicating the onset of division of the heart into two chambers: the atrium (ventral) and the ventricle (dorsal).

During S4 the pronephric ducts begin to extend posteriorly from the kidneys, running ventrally to the somites. Beneath the notochord, a thick rod of endoderm lies directly over the yolk. At the start of S4 this is only visible if the embryo is removed from the yolk, but by the end of the step the expansion of the pericardial chamber reveals the presence of a deep keel of endodermal tissue beneath the anterior-most somites. This will develop into the stomach.

In some embryos the first signs of branchial differentiation may be visible ventral to the otic vesicle. The mandible also begins to develop as an upside-down 'V' beneath the midbrain-hindbrain boundary.

Step 17 (S5) - Tailbud freeing from yolk (Fig. 2.2)
22 - 24 days (171 - 187 points)
32 - 40 somites

The posterior end of the tailbud is no longer continuous with the yolk surface, but has become slightly undercut at the start of step S5. By the end of the step, the tailbud is free of the yolk almost to the level of the last somite.

The heart becomes more cylindrical, and is inclined anteriorly at its middle. Approximately halfway through step S5, when the embryo has around 35 somites, the heart begins to move in a gentle rocking motion, forward and back. By the end of the step, blood cells may be visible in the heart, being pushed upward when the heart moves anteriorly and then dropping back as the heart returns to its original position. This circulation does not extend beyond the heart itself. The timing of the onset of heartbeat depends on temperature (see *Effects of temperature...*, p. 96).

The gut becomes more easily visible, and a developing lumen can be seen posterior to, but not in, the stomach primordium. The liver starts to develop as a mass of undifferentiated tissue on the surface of the yolk, joined to the posterior-ventral region of the stomach primordium at somites 7 - 9. The pronephri have increased in size and now bend back ventrally in a C-shape. By the end of the step the rudiment of the operculum has appeared as a vertical protruding ridge ventral to the front of the otic vesicle. Posterior to this, the first branchial arch appears. Towards the end of S5, condensations of tissue develop lateral to somites 3 - 5, borne on the surface of the swollen pericardial chamber. These are the primordia of the pectoral fins.

The developing lens begins to separate from the surrounding optic cup. With the exception of the first two or three, more and more somites become chevron-shaped, reaching back to somite 30 by the end of the step. The appearance of the notochord changes in an anterior-posterior sequence as some of its cells develop large vacuoles; this change reaches as far back as somite 10 by the end of S5.

Step 18 (S6) - Finfold (Fig. 2.2)
24 - 26 days (189 - 207 points)
39 - 48 somites

The embryonic finfold develops; it appears first at the beginning of S6, dorsal to the most posterior somites, where the narrowest part of the body reaches the swollen tailbud. By the end of step S6 it runs dorsally from the back of the hindbrain to the end of the tailbud, and is also present ventrally posterior to the developing anus. As new somites are produced, more and more of the tailbud becomes free from the yolk; by the end of S6 as many as 16 somites are free.

The heart gradually bends forward so that by the end of the step the atrium lies horizontally, on top of the yolk, while the ventricle leans slightly posteriorly as it joins the embryo. The atrium also begins to move towards the left side of the embryo. The simple rocking motion seen previously changes to a posterior-anterior wave of contraction, initially confined to the atrium. It is powerful enough to propel blood cells beyond the heart and into the paired dorsal arteries and then the dorsal aorta

(DA), which begins to differentiate from the dorsal part of the intermediate mass, lying beneath the notochord. By the end of the step the intermediate mass has differentiated as far back as somite 35, where blood cells traverse the gut and return anteriorly over the surface of the yolk beneath the embryo, across the bottom of the pericardial chamber, and reenter the atrium. (Note: This study chronicles the development of circulatory patterns; see Isogai & Horiguchi (1997) for a description of the formation of the blood vessels prior to circulation.)

The ventral ends of somites 2 - 4 expand laterally into the developing pectoral fins. Two distinct branchial arches are present posterior to the operculum. The pronephric ducts extend all the way to the developing anus at somite 37, where they bend ventrally. Lumina are present in the ducts, and in the gut also, for almost all of their length.

Step 19 (S7) - Olfactory placodes (Fig. 2.2)
26 - 28 days (205 - 220 points)
47 - 56 somites

Step S7 commences as the circular olfactory placodes develop in front of the eyes, a little above the surface of the yolk.

At the beginning of this step, the blood flow in the DA turns ventrally at somite 35, and some of it continues to do so throughout the step. However, the DA slowly extends posteriorly, carrying most of the blood flow as far back as somite 40 (approximately) by the end of the step. (Posterior to the anus, the DA is called the caudal artery). This flow then returns anteriorly along the caudal vein. At the anus, it combines with the blood that left the DA at somite 35, traverses the gut through the anal vein, and continues anteriorly along the intestinal vein. At the same time that the DA is extending, the vitelline vein (VV) that connects the intestinal vein to the heart is moving out onto the left side of the yolk - this is the first step in the vascularisation of the yolksac.

A lumen begins to develop along the dorsal part of the stomach primordium, eventually joining with that in the rest of the gut. A branch of this lumen also extends ventrally into the main mass of the stomach primordium. The third branchial arch forms, and in some embryos the fourth begins to develop, although this usually does not occur until step S8. Meanwhile, a cavity appears between the first arch and the operculum. The snout becomes undercut as the head begins to separate from the yolk. By the end of this step the pectoral fins are about one third to one half as tall as they are wide (in side view), and as many as 30 somites are free from the yolk.

At 6°C, the embryo begins to exhibit spontaneous contractions of the trunk muscles during this step. It also responds to touch stimulus; by the end of the step, muscle contractions can be seen in the first 20 - 25 somites. The development of trunk muscle activity is also affected by temperature (see *Effects of temperature...*, p. 96)

Step 20 (S8) - Otolith nuclei (Fig. 2.2)
28 - 30 days (221 - 239 points)
55 - 64 somites

Step S8 begins with the formation of small otolith 'nuclei' in the otic vesicle, at the ventral-anterior corner, attached to the vesicle wall by tiny hair-like projections. These are best viewed against bright field illumination. At the end of S8, an embryo rolled onto its back will twitch from side to side as if trying to right itself, suggesting the development of a sense of balance.

The VV continues to encompass an increasing area of the yolk surface. The exact proportion is difficult to determine, as the vein is not yet visible through the chorion, and dechorionation invariably ruptures the yolk. However, it does not yet extend past the region of densely-packed oil droplets beneath the embryo. The caudal artery continues to extend posteriorly, reaching somite 44 by the end of the step. (The most posterior vacuolised cell in the notochord is now usually found at the same position as the tip of the caudal artery.) The 'short cut' from the DA to the anal vein at somite 35 disappears. Some of the blood from the intestinal vein bypasses the VV and passes forward under the gut along the hepatic vein, turning ventrally at the liver

out onto the left side of the yolk sac, eventually joining the VV. A small flow of blood can now be seen exiting the DA ventrally and circulating in the region of the pronephri - this indicates the initial development of the hepatic artery. Step S8 ends with the establishment of circulation in the paired internal carotid arteries. These carry blood forward from the heart, around the circumference of the optic cup and into the anterior cardinal veins (medial to the otic vesicles), before re-entering the heart.

The pectoral fins are from 1/2 to 2/3 tall as wide. The urinary bladder forms at the ends of the pronephric ducts, just posterior to the developing anus. As many as 40 somites are free of the yolk.

The final number of somites formed is 59 - 66, depending partly on environmental temperature (see *Effects of temperature...*, p. 96). Somites in excess of 61 - 62 are formed at a slightly lower rate, so that in some embryos somitogenesis continues into the next phase.

*Yolksac vascularisation phase (Fig. 2.3, p. 86):*

The process of yolksac vascularisation, the envelopment of the yolk in a complex mesh of blood vessels, actually begins during step S7 and often continues into the caudal fin ray phase. For convenience, however, the term is used to describe the interval of development between the end of regular somite formation and the formation of the first caudal fin ray.

Step 21 (Y1) - Eye Pigment (Fig. 2.3)
30 - 33 days (236 - 258 points)
Up to 2/5 of the yolksac (YS) vascularised

Step Y1 begins with the appearance of a thin dusting of brown pigment at the dorsal rim of the optic cup. This band of pigment extends so that by the end of the step it covers roughly 2/3 of the circumference of the eye. The ends of the C-shaped optic cup turn inwards at the choroid fissure.

The posterior extent of the caudal artery increases from somite 44 to 53. The hepatic artery is completed and carries blood directly from the DA to the liver. From there it travels out onto the right side of the yolksac via a small number of blood vessels which ultimately join with the VV. An increasing flow of blood from the intestinal vein avoids entering the VV directly, passing instead under the embryo along the hepatic vein to the liver, where it passes onto the yolksac.

Branches from the carotid arteries appear at the midbrain-hindbrain boundary, ascending medially and then descending laterally to join the anterior cardinal veins. At the end of Y1 the appearance of hemoglobin lends the blood a light pink colour, although this is visible only where the circulation is strongest, in the VV and the heart.

After the final somite is formed, incomplete vertical furrows appear in the undifferentiated tissue of the tail bud, forming 'false somites'. The tailbud begins to develop into the caudal fin - mesenchyme accumulates posterior to the anus and extends posteriorly, becoming thickest beneath the last somites, which elongate ventrally. A thin dorsal layer of mesenchyme appears slightly later, extending across the top of all but the last few somites. The caudal tip of the notochord may flex dorsally, although the timing of this event is quite variable.

The size of the pectoral fins increases progressively, so that by the end of the step they are slightly taller than they are wide; also, each fin is wider at its middle than where it is connected to the embryo's body. The head becomes free from the yolk as far back as the upper jaw.

Step 22 (Y2) - Gut Capillaries (Fig. 2.3)
33 - 36 days (262 - 282 points)
1/3 - 2/3 of the yolksac vascularised

During this step the circulatory system undergoes major changes. A new branch from the DA appears at somite 30 - 33, carrying blood to the newly-formed intestinal artery. This artery carries blood both anteriorly and posteriorly above the gut, connecting to the intestinal vein by a number of gut capillaries on either side of

the intestine. More anteriorly, a vessel branching from the hepatic artery carries blood back along the gut, but it traverses the intestine and ultimately joins the hepatic vein, without connecting to the intestinal artery. As the VV extends onto the yolk, it produces subsidiary veins that branch out over both sides of the yolk sac before rejoining the VV.

The blood returning from the tail along the caudal vein no longer enters the intestinal vein via the anal vein; instead, it continues forward just beneath the DA, in the median axial vein (MAV). At the position of the pectoral fins, this divides into the paired posterior cardinal veins, each of which then splits into two branches, one running either side of the pronephros. These branches then reunite before joining with the anterior cardinals at the ducts of Cuvier, which connect to the atrium of the heart just anterior to the left pectoral fin. At the beginning of Y2, some blood from the intestinal vein escapes into the now-redundant anal vein and is carried up to the MAV, the reverse direction of the original flow; but by the end of the step the anal vein has usually disappeared. Blood ascending from the heart through the first pair of aortic arches (i.e. the mandibular arches) can now be clearly seen through a 'window' in the operculum, and the caudal artery extends back as far as somite 57.

All of the tissue of the tailbud is now differentiating. The dorsal mesenchyme reaches almost to the tip of the tail, and the caudal portion of the ventral finfold begins to expand. The rim of dark pigment around the optic cup continues to extend until it reaches the margins of the choroid fissure. Pigment also begins to spread out onto the dorsal surface of the eye. The pectoral fins incline posteriorly. The head becomes free of the yolk as far back as the lower jaw.

Step 23 (Y3) - Semicircular Canals (Fig. 2.3)
36 - 39 days (284 - 304 points)
2/3 - 3/4 of the yolksac vascularised

Primordia of three semicircular canals develop in each otic vesicle at the beginning of Y3. The most ventral canal often appears slightly in advance of the

dorsal pair. A scattering of small red pigment cells become visible over the otic vesicle, and dark pigment spreads over the entire optic cup.

Circulation appears almost simultaneously at the first two pairs of branchial arches (i.e. in aortic arches 3 and 4). (For a more detailed description of the development of circulation in salmonid gills, see Vernier (1969)). The pink colour of the blood is now apparent in all major vessels. An increasing proportion of the blood in the intestinal vein enters the hepatic vein rather than passing directly into the VV. The hepatic vein passes to the left of the gut rather than beneath it, while the liver is located entirely on the right side of the embryo. The caudal artery, and notochord vacuolisation, reach to the last somite by the end of this step.

The first deep intersegmental blood vessels appear during this step. These capillaries alternately ascend from the DA and descend to the MAV at the boundaries between successive somites, adjacent to the notochord and neural tube. Paired blood vessels on either side of the top of the neural tube connect the dorsal ends of the intersegmental blood vessels, which develop in a highly imperfect anterior-posterior sequence. At the end of Y3 the most posterior intersegmental vessel was observed between somites 44 and 45, although the position varied considerably between embryos, and even between the left and right side of the same embryo. Also, a new blood vessel returning posteriorly from the brain becomes paired behind the position of the otic vesicle, and produces loops which connect to both the anterior cardinals and the first intersegmentals.

The head is free to approximately halfway between the lower jaw and the operculum. The 'false somites' formed previously gradually become less distinct as the caudal fin differentiates further. Both the dorsal and ventral caudal finfold are now expanding to form a broad flange. At the end of this step, the ventral mesenchyme at somites 40 - 46 has grown denser, and is taller than the surrounding mesenchyme, as the anal fin begins to develop.

Step 24 (Y4) - Body Pigment (Fig. 2.3)

39 - 42 days (309 - 327 points)

3/5 - 7/8 of the yolksac vascularised

Black, stellate melanophores appear along the tops of the most anterior somites at the beginning of step Y4. They increase in number and size while also extending posteriorly, reaching as far back as somite 25 in some embryos by the end of the step. Small, non-stellate melanophores also appear on the surface of the yolksac lateral to the gut, and above the hindbrain.

All the flow from the intestinal vein now passes into the hepatic vein, then across the liver and onto the yolk sac, eventually collecting into the VV. Branches of the internal carotid arteries become visible at the front of the head, where they emerge to the surface and then join the vessels running back over the tops of the eyes. Vessels branching from this optic circulatory system begin to branch dorsally, over the front of the forebrain. At the same time, short, dead-end branches develop from the vessels at the midbrain-hindbrain boundary, extending onto the dorsal surface of the forebrain. The posterior end of the branch from the hepatic artery that runs above the gut connects with the anterior end of the intestinal artery, producing a small region of ebb-and-flow circulation at the site of connection, between somites 18 - 20.

Numerous actinotrichia, fine supporting ray-like structures (not to be confused with the much larger fin rays that develop later), appear in the ventral caudal finfold. The dorsal fin begins to develop at somites 23 - 28, as the mesenchymous tissue becomes denser and taller than the surrounding tissue. The finfold at the dorsal and anal fins expands at the end of Y4.

The head is free of the yolk as far back as the operculum, exposing the heart. The most anterior branchial arches begin to develop into gill slits. Each pectoral fin is twice as wide in its middle as at its base, expanding posteriorly. The embryo can now be quite easily removed from the chorion without rupturing the yolksac.

Step 25 (Y5) - Circulation over Forebrain (Fig. 2.3)

42 - 45 days (333 - 351 points)

3/4 - >9/10 of the yolksac vascularised

The vessels developing at the front of the forebrain make connections to the descending vessels at the midbrain-hindbrain boundary, forming a functional plexus over the forebrain. By the end of Y5 they have usually also connected to the branches at the top of the forebrain that began to develop in Y4. Circulation develops at the third and then the fourth branchial arch (aortic arches 5 and 6), and in the hyoid arch (aortic arch 2, behind the mandibular arch). Intersegmental blood vessels are present to somite 55 - 60. Blood may be present (but not circulating) in the developing subclavian veins, which run from the posterior cardinal vein into the pectoral fins, along a loop concentric to the edge of the fin, and then rejoin the cardinals just before they enter the heart.

The caudal artery reaches almost to the tip of the tail; but in most embryos a 'bypass' develops at somites 57 - 58, carrying most of the blood from the caudal artery to the caudal vein, bypassing the developing caudal fin. Also, at 6°C, bile production begins in the hindgut, appearing near the anus first. Its presence is indicated by a light tinge of green. The operculum begins to cover the first branchial arch. The notochord becomes vacuolised all the way to the caudal tip.

Step 26 (Y6) - Anal Fin Musculature (Fig. 2.3)

45 - 48 days (356 - 375 points)

>9/10 - All of the yolksac vascularised

The ventral edges of the somites above the developing anal fin produce short, muscular buds penetrating the thick mass of mesenchyme. A similar process occurs at the dorsal fin. By the end of Y6, approximately 3 anal somites and 4 dorsal somites have developed these extensions.

Deep intersegmental vessels are present between all somites, and melanophores appear over the dorsal region of the otic vesicle. The angle between the cerebellum and the rest of the hindbrain (referred to henceforth as the 'hindbrain

angle) begins to decrease as the cerebellum starts to slope posteriorly. At the end of this step the membrane covering the mouth of the embryo has been resorbed, and in most eggs the entire yolksac is vascularised.

*Caudal fin ray phase (Fig. 2.4, p. 87):*

This phase begins with formation of the first caudal fin ray; the process of fin ray production continues past hatching to step R2.

Step 27 (F1) - Caudal Fin Rays (Fig. 2.4)
48 - 52 days (380 - 413 points)
1 - 4 Caudal Fin (CF) rays

The mesenchyme in the caudal fin begins to segregate into short rays perpendicular to the end of the notochord, which is at about 30° to the main body axis. These rays increase steadily in both number and length. The first rays are formed in the centre of the caudal fin, with additional rays being added anteriorly and posteriorly (or ventrally and dorsally, as the angle of the notochord and of existing rays changes).

By the end of the step, blood is often present, but not yet circulating, in the hypural region of the caudal fin; it usually fills a Y-shaped space at the largest fin ray. A new set of intersegmental blood vessels begins to develop, running along the dorsal intersomitic boundaries at the surface and connecting each of the original, deep intersegmentals with those adjacent. As with the deep vessels, flow in these superficial intersegmentals alternates between ascending and descending between subsequent somites. A new vessel branches posteriorly from the descending vessels at the midbrain-hindbrain boundary, carrying blood dorsally over the otic vesicle to the anterior cardinal veins. The blood in the subclavian vein begins to circulate weakly, and at 6°C the pectoral fin muscles twitch sporadically. (The circulation and movement often begin earlier on one side of the embryo, usually the left.)

Embryos have as many as 5 muscular buds in the anal fin and 8 in the dorsal fin. The dorsal finfold begins to expand at somites 43 - 50, the site of the future adipose fin. The hindbrain angle decreases from approximately 70° to as little as 30°, reducing the size of the space above the hindbrain. The operculum covers all of the first branchial arch, and begins to extend over the second. Melanophores appear over the forebrain, and may extend past the back of the dorsal fin.

Step 28 (F2) - Pelvic Fins (Fig. 2.4)
52 - 55 days (408 - 435 points)
2 - 5 CF rays

Mats of tissue appear ventral to somites 25 - 27 - these are the rudiments of the pelvic fins. By the end of step C2, they are approximately 1/4 - 1/3 the depth of the ventral finfold.

The 'bypass' loop between the caudal artery and vein expands and begins to loop out into the hypural region of the caudal fin, towards the fin rays, which are now up to 1/3 the depth of the caudal fin itself. Embryos have up to 6 anal and 10 dorsal muscular buds. Body pigmentation continues to become denser and extends further back, past the anus. It also starts to develop at the ventral edges of the somites and over the gut.

The hindbrain angle decreases below 30°. The ends of the optic cup have become pressed flat against each other. The shape of the anteriormost somites changes from a simple chevron to a W-shape as the dorsal and ventral edges curve forwards.

Step 29 (F3) - Caudal Fin Circulation (Fig. 2.4)
55 - 58 days (429 - 457 points)
3 - 6 CF rays

The loop of the caudal artery produces several radial arterioles that pass between the fin rays and then return along radial venules to rejoin the caudal vein.

Capillaries develop midway along the subclavian vein, projecting dorsally into the pectoral fin. Stubby projections appear at the edge of branchial arch 1, initially at the most ventral position and then spreading dorsally. Similar projections appear on arch 2 shortly afterwards. These will develop into the gill filaments. The operculum continues to progress posteriorly, covering most or all of the second branchial arch by the end of the step.

The number of muscular buds in the bases of the anal and dorsal fins stabilises at approximately 7 and 10 respectively. The size of the pelvic fins increases from 1/3 to 1/2 the depth of the ventral finfold. The dorsal fin, which previously has been symmetrical in the anterior-posterior direction, now begins to slope gently backwards.

Body pigment extends posteriorly past the anal fin and developing adipose fin. As melanophores appear on the sides of the embryo, they usually line up along the edges of the somites, producing a segmented pattern of pigmentation. Black pigment cells also become visible on the dorsal finfold, and become denser above the anal fin.

At the end of F3 the ends of the optic cup begin to fuse at the top of the choroid fissure.

Step 30 (F4) - Pigmentation at the Most Posterior Somite (Fig. 2.4)
58 - 62 days (455 - 486 points)
5 - 8 CF rays

Step F4 begins with the appearance of melanophores at the hindmost somites, both dorsally and ventrally. They soon form a dense cluster, and continue to extend posteriorly along the notochord. By the end of the step melanophores are developing on the main part of the caudal fin, in the region of the fin rays. The depth of the developing pelvic fins is approximately 1/2 - 2/3 that of the ventral finfold.

Superficial intersegmentals may be present at the most posterior somites. Capillaries develop in the ventral region of the first branchial arch, and then at the second, extending from the aortic arches into the developing gill filaments. The

rudiments of filaments begin to develop at the third branchial arch. The operculum covers all of branchial arch 2 at the onset of this step, and progresses to cover most of the third arch. By the end of this step, the hindbrain angle is reduced to 0° - the cerebellum lies flat against the rest of the hindbrain.

Step 31 (F5) - Anal Fin Loses Symmetry (Fig. 2.4)
62 - 66 days (480 - 520 points)
6 - 10 CF rays

The anal fin begins to incline posteriorly at the beginning of step F5. The angle at the back of the dorsal fin (relative to the midline) increases from 30° to 60°.

Also at the beginning of F5, a 'step' develops in the MAV, just posterior to the back of the dorsal fin; anterior to this, the MAV is separated from the DA by a thickened region of tissue. Capillaries extend into the more ventral filaments of the third branchial arch, at the same time as filaments develop at the fourth arch. The operculum usually covers all of the third arch and some of the fourth by the end of this step, but this becomes increasingly difficult to judge, being affected by the angle of the embryo's head and which side is examined.

Just opposite the caudal tip of the notochord, an indentation appears in the edge of the caudal fin. The pelvic fins continue to increase in size, almost reaching the ventral edge of the finfold by the end of F5. Melanophores develop over the central region of the operculum; they also become increasingly concentrated over the forebrain, the otic vesicle, and above the bases of the pectoral fins. The mesenchyme in the dorsal finfold forms dense accumulations at the adipose fin and above the caudal fin. The head of the embryo begins to nod up and down in a gulping movement, and at 6°C the pectoral fins are now beating for most of the time, although in an uncoordinated manner.

Step 32 (F6) - Formation of Dorsal Fin Rays Begins (Fig. 2.4)

66 - 72 days (510 - 562 points)

7 - 14 CF rays

The mesenchyme at the centre of the dorsal fin begins to differentiate, forming the rudiments of up to 3 fin rays approximately 1/5 - 1/3 the height of the fin itself. The angle at the back of the fin increases to as much as 80°. The pelvic fins reach the edge of the ventral finfold at the beginning of this step, and continue to expand ventrally. The angle at the back of the anal fin is approximately 30°.

Capillaries are present in all the filaments on the first two branchial arches, and develop in the most ventral filaments on the fourth. New deep intersegmental vessels appear, ventral counterparts to the existing dorsal vessels. These are visible at the anal fin, but not elsewhere. The lens, which has previously always been lighter in colour than the surrounding optic cup, begins to darken conspicuously. A new patch of pigment begins to develop between the back of the eye and the operculum. At 6°C, the gulping motions of the head and the beating of the pectoral fins become synchronised.

Step 33 (F7) - Caudal Artery Connects to Caudal Fin Plexus (Fig. 2.4)

72 - 77 days (551 - 612 points)

11 - 16 CF rays

The tip of the caudal artery connects to the complex of blood vessels among the fin rays; the original posterior end of the caudal vein disappears.

Embryos possess from 4 to 8 dorsal fin rays, which have developed a light green tinge. The front of the dorsal fin develops a distinct hump at the base, and the angle at the back of the fin increases above 90°. Resorption of the dorsal finfold (DFF) begins directly behind the dorsal fin, so that by the end of F7 there is a distinct notch.

Anal fin rays develop midway through step F7, and by the end of the step up to 5 rays are present, 1/3 - 3/5 the depth of the fin itself. Pectoral fin rays begin to

develop at the same time in the region of the subclavian vein. The pelvic fins swell so that they are now wider at their middles than at their bases. The pigment cells on the caudal fin begin to align along the centremost fin rays. The lens becomes as dark as, if not darker than, the optic cup, the ends of which have fused for more than half of the original choroid fissure. The inner ventral edge of the olfactory organ begins to project into the olfactory cavity. At 6°C, some embryos begin to hatch, tail first.

Step 34 (F8) - Formation of Pelvic Fin Rays Begins (Fig. 2.4)
77 - 82 days (606 - 645 points)
14 - 17 CF rays

The beginning of step F8 is marked by the formation of the first rays in the pelvic fins; approximately three rays can be seen to be forming by the end of the step.

As the anal fin becomes increasingly inclined posteriorly, the angle of its posterior margin relative to the midline exceeds 90°. Embryos possess 7 - 10 dorsal and 4 - 9 anal fin rays. During this step the angles at the backs of the dorsal and adipose fins increase from approximately 100° to 135° and from 15 to 30° respectively. Up to six rays are present in the pectoral fins, and the capillaries branching from the subclavian vein become organised so that they run parallel to these rays.

Melanophores may appear under the eye, on the lower jaw, on the anal fin, and at the bases of the pelvic fins, but the timing of these events is highly variable. The ends of the optic cup are fused for all but the most ventral region of the former choroid fissure. At 6°C, all embryos have hatched by midway through this step.

*Finfold resorption phase (Fig. 2.5, p. 88):*

Resorption of the dorsal finfold actually begins during the previous phase, just behind the head, but initially progresses very slowly. After step F8, however, the pace of resorption quickens noticeably. The disappearance of the last trace of the median finfold broadly coincides with the timing of first feeding.

Step 35 (R1) - Caudal Fin Ray Segments (Fig. 2.5)
82 - 87 days (650 - 684 points)
16 - 19 CF rays

The first row of segments is formed in the caudal fin. The first 11 - 12 segments appear almost simultaneously, 3/5 - 3/4 along the fin rays. They appear at the more central fin rays first; later segments are added dorsally and ventrally. By the end of R1, 14 - 15 segments are present.

Up to 5 rays are present in each of the pelvic fins. The dorsal and anal fins have 10 - 12 and 7 - 9 fin rays respectively, approaching their final number. The posterior angle of the dorsal fin stabilises at approximately 150 - 160°. The angle at the back of the adipose fin increases to more than 60°. Up to 9 fin rays are present in the pectoral fins. The DFF immediately behind the back of the dorsal fin disappears, although this region of resorption does not yet extend past the most posterior margin of the dorsal fin. The proportion of the anterior DFF (i.e. between the position above the front of the yolk sac and the dorsal fin) which has been resorbed increases, from 1/5 at the beginning of R1 to as much as 1/2.

The tissue in the DFF posterior to the adipose begins to form a distinct ridge, approximately 2/3 the height of the finfold. Branchiostegal rays appear in the ventral portion of the operculum. The first one to four such rays become visible quite rapidly, but later, more ventral rays are added more slowly, at a regular rate. At the end of step R1, blood can be seen circulating in one or more small blood vessels in the adipose fin in most free embryos.

Step 36 (R2) - Dorsal Fin Becomes Serrated (Fig. 2.5)

87 - 94 days (684 - 753 points)

At the beginning of R2, the margin of the dorsal fin becomes serrated between the tips of the anterior-most fin rays. This serration will slowly progress along the entire dorsal margin of the fin. A similar process begins in the anal fin, and at the centre of the caudal fin margin, near the end of the step. The angle of the posterior margin of the adipose fin increases to 90°; that of the anal fin also increases until the 'posterior' margin of the fin is almost horizontal. The extent to which the anterior DFF has been resorbed increases rapidly from 1/2 to as much as 7/8. The posterior DFF is resorbed past the hindmost margin of the dorsal fin approximately midway through the step, and halfway to the front of the adipose by the end of the step. The finfold between the adipose and caudal fins, above the ridge formed in R1, also disappears. A similar ridge develops in the finfold behind the anal fin.

Fin ray production is now completed in the dorsal, anal, and pectoral fins, which have approximately 13, 10 and 10 rays. The main (or primary) fin rays in the caudal fin have also reached their final number of approximately 20. Midway through R2, a second row of 8 - 12 segments appears in the caudal fin, which is expanding rapidly. The portion of the fin posterior to the indentation formed in step F5 (i.e. posterior to the tip of the notochord) becomes as tall as the more anterior portion. Between two and six branchiostegal rays are visible. Melanophores develop on the branchial arches. At the end of R2, the maxillary flap on the upper jaw begins to extend posteriorly.

Step 37 (R3) - Anterior Dorsal Finfold Fully Resorbed (Fig. 2.5)

94 - 102 days (750 - 816 points)

All of the DFF in front of the dorsal fin has been resorbed. This is swiftly followed by resorption of the remainder of the DFF behind the dorsal fin, and also of the ventral finfold behind the anal fin. Resorption of the pre-anal ventral finfold begins at the back of the yolk sac, although its progress is slow, and often obscured by the pelvic fins.

The angle at the back of the adipose fin increases from 90° to more than 120°; as the fin extends more posteriorly, it may become partially undercut. The caudal fin becomes taller behind than in front of the notochord. At the same time, production of procurrent caudal fin rays begins, in the ventral portion of the fin that extends onto the caudal peduncle. These rays are distinctly shorter in their final length than primary rays, and are also produced at a much slower rate. Segments do not develop at these rays. The shape of the posterior margin of the caudal fin (which is now fully rippled) changes from being convex to slightly concave as the fork begins to develop.

Between 5 and 7 branchiostegal rays are present. Having remained stable at approximately 16 during R2, the number of segments in the first row in the caudal fin increases to approximately 18. The second row also stabilises temporarily at 16; and at the end of the step, up to 6 segments appear in a third row.

Step 38 (R4) - Parr Marks (Fig. 2.5)
102 - 109 days (814 - 865 points)

The melanophores on the sides of the free embryo congregate into oval-shaped parr marks. Up to five of these markings appear above the yolk sac and pelvic fins. During this step, parr marks are normally confined to the pre-anal region of the body, but post-anal parr marks may begin to develop at the end of this step or at any time during the next step.

Midway through R4, 6 - 8 segments appear in the dorsal fin. The number of segments in the caudal fin increase to approximately 19, 16 and 12 in the three rows. What was the posterior margin of the adipose fin now lies almost horizontally. The pectoral fins adopt a more horizontal position, so that their uppermost margins are level with the midline or even with the top of the yolk sac. The pre-anal finfold is resorbed to, or past, the bases of the pelvic fins.

Two ventral procurrent caudal fin rays (V.P.C. fin rays) and between 6 and 8 branchiostegal rays are present. Production of dorsal procurrent caudal fin rays begins, in the dorsal part of the caudal fin anterior to the notochord.

Step 39 (R5) - Anal Fin Ray Segments (Fig. 2.5)

109 - 117 days (861 - 919 points)

Between four and six segments appear in the anal fin, the number increasing up to 9 during the step. Approximately 19, 17 and 13-16 segments are present in the first three rows in the caudal fin; at the end of the step (or occasionally at the beginning of the next step) up to five segments develop in a fourth row.

The pre-anal finfold may be resorbed as far back as the posterior tips of the pelvic fins. Between two and four V.P.C. fin rays and 7 - 9 branchiostegal rays are present. The beginnings of the 'button-up' scar become visible near the back of the yolksac.

Step 40 (R6) - Second Row of Dorsal Fin Ray Segments (Fig. 2.5)

117 - 127 days (913 - 1010 points)

Between one and five segments appear in a second row in the dorsal fin at the beginning of step R6. Midway through the step, a similar number develop in a second row in the anal fin. The pre-anal finfold is reduced to a small flap shallower than the anus. Between three and four procurrent fin rays are now visible in the dorsal part of the caudal fin; three to five V.P.C. fin rays, and 8 - 10 branchiostegal rays are also present.

The step is completed with the appearance of a fifth row of caudal fin segments, a third row of dorsal fin segments, and the complete resorption of the pre-anal finfold. Depending on temperature (see Chapter 3), yolk absorption is completed, and the vitelline vein and capillaries cease to be visible.

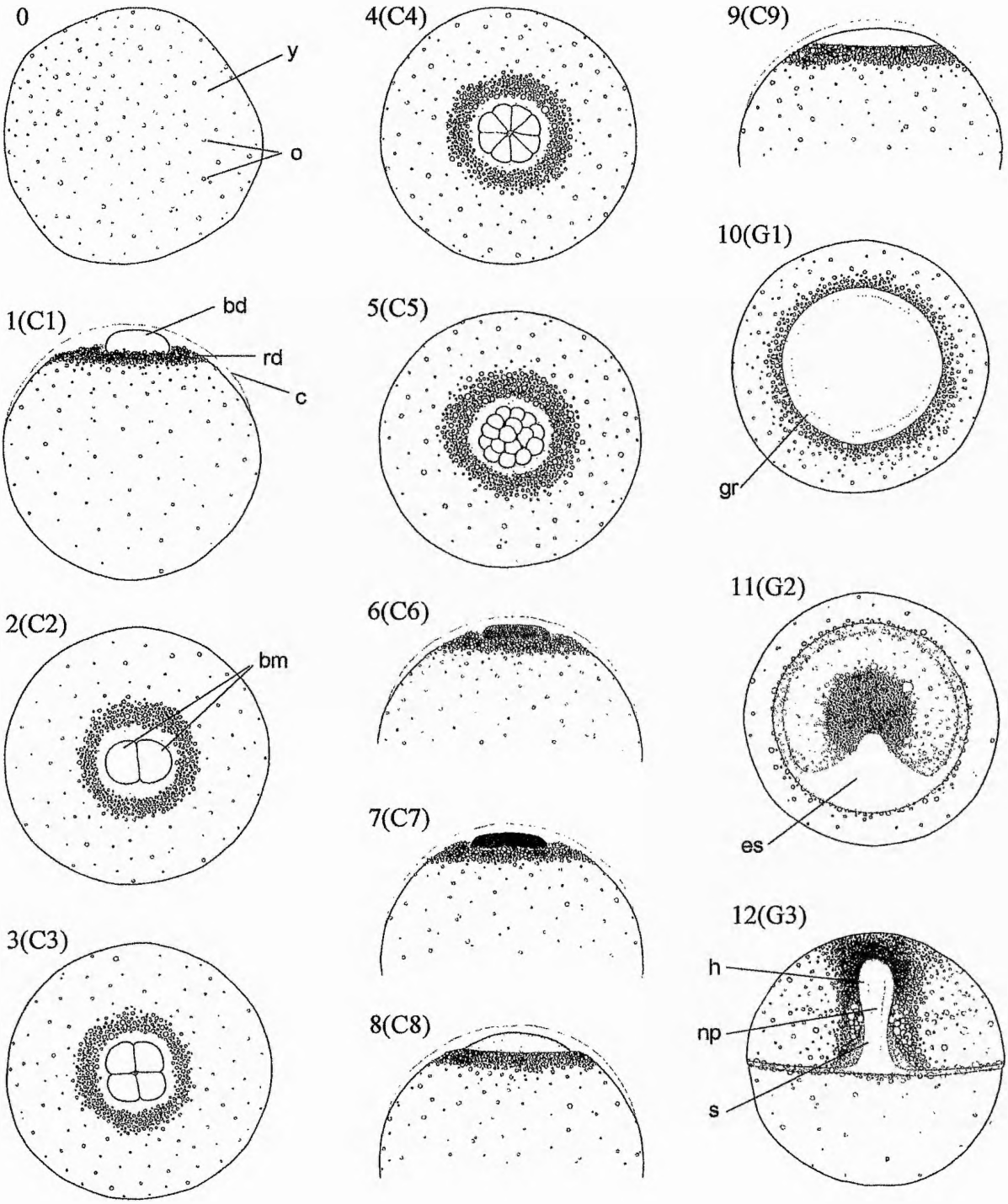



**Fig. 2.1.** Cleavage (C) and Gastrula (G) phases : Steps 0 - 12. Drawings represent embryos at the end of each step. Steps 2, 3, 4, 5, 10 and 11 are depicted from top view; Steps 0, 1, 6, 7, 8, 9 and 12 are depicted from side view. bd = blastodisc; bm = blastomeres; c = chorion; es = embryonic shield; gr = germ ring; h = head; np = neural plate; o = oil droplets; rd = ring of oil droplets; s = site of formation of first somites; y = yolk.

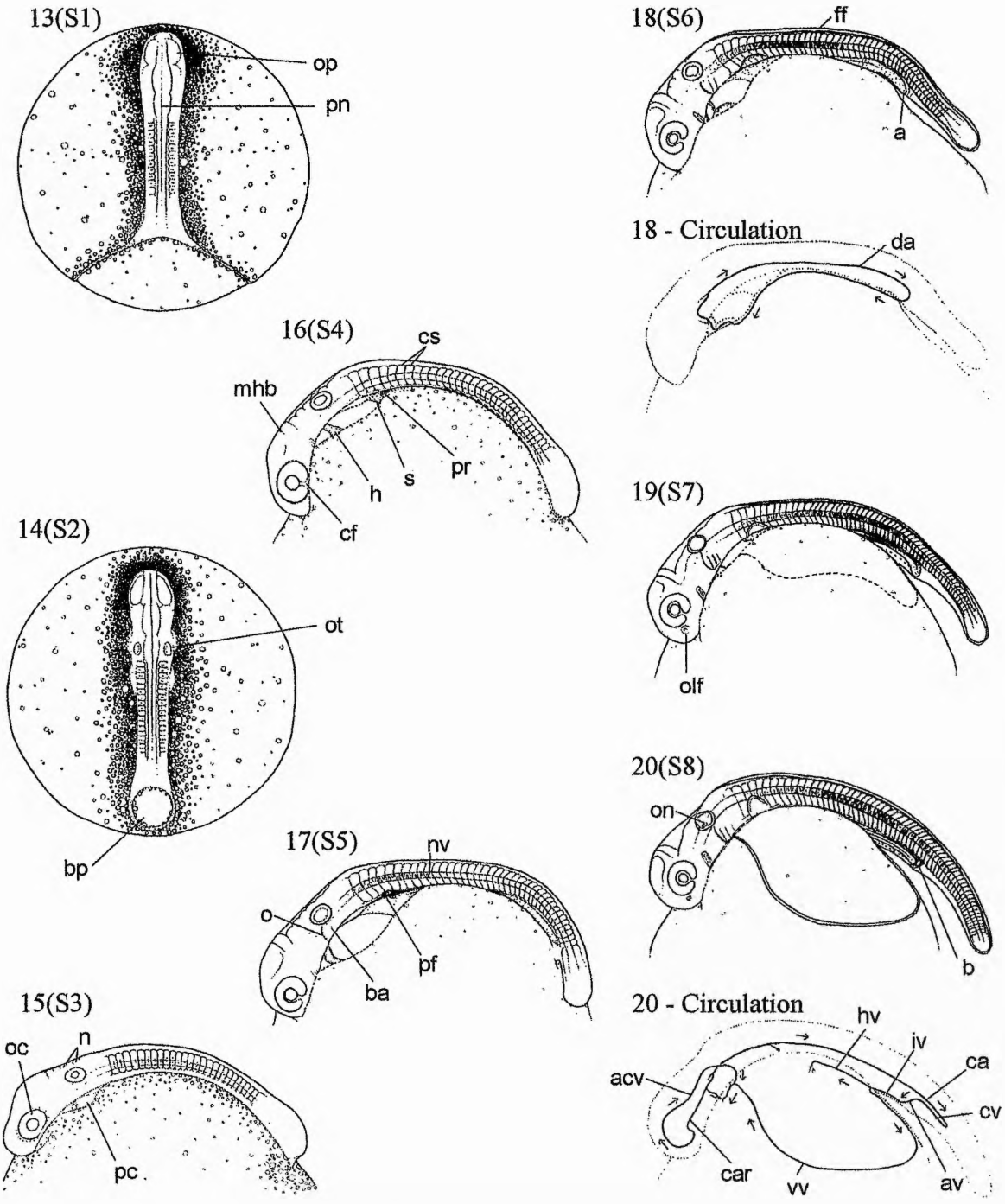



**Fig. 2.2.** Somitogenesis (S) phase : Steps 13 - 20. Drawings represent embryos (side view) at the end of each step. a = future anus; acv = anterior cardinal vein; av = anal vein; b = bladder; ba = branchial arch; bp = blastopore; ca = caudal artery; car = carotid artery; cf = choroid fissure; cs = chevron-shaped somites; cv = caudal vein; da = dorsal aorta; ff = finfold; h = heart; hv = hepatic vein; iv = intestinal vein; mhb = midbrain-hindbrain boundary; n = neuromeres; nv = notochord vacuolisation; o = operculum; oc = optic cup; olf = olfactory placodes; on = otolith nuclei; op = optic primordia; ot = otic placodes; pc = pericardial chamber; pf = pectoral fins; pn = primary neurulation; pr = pronephri; s = stomach; vv = vitelline vein.

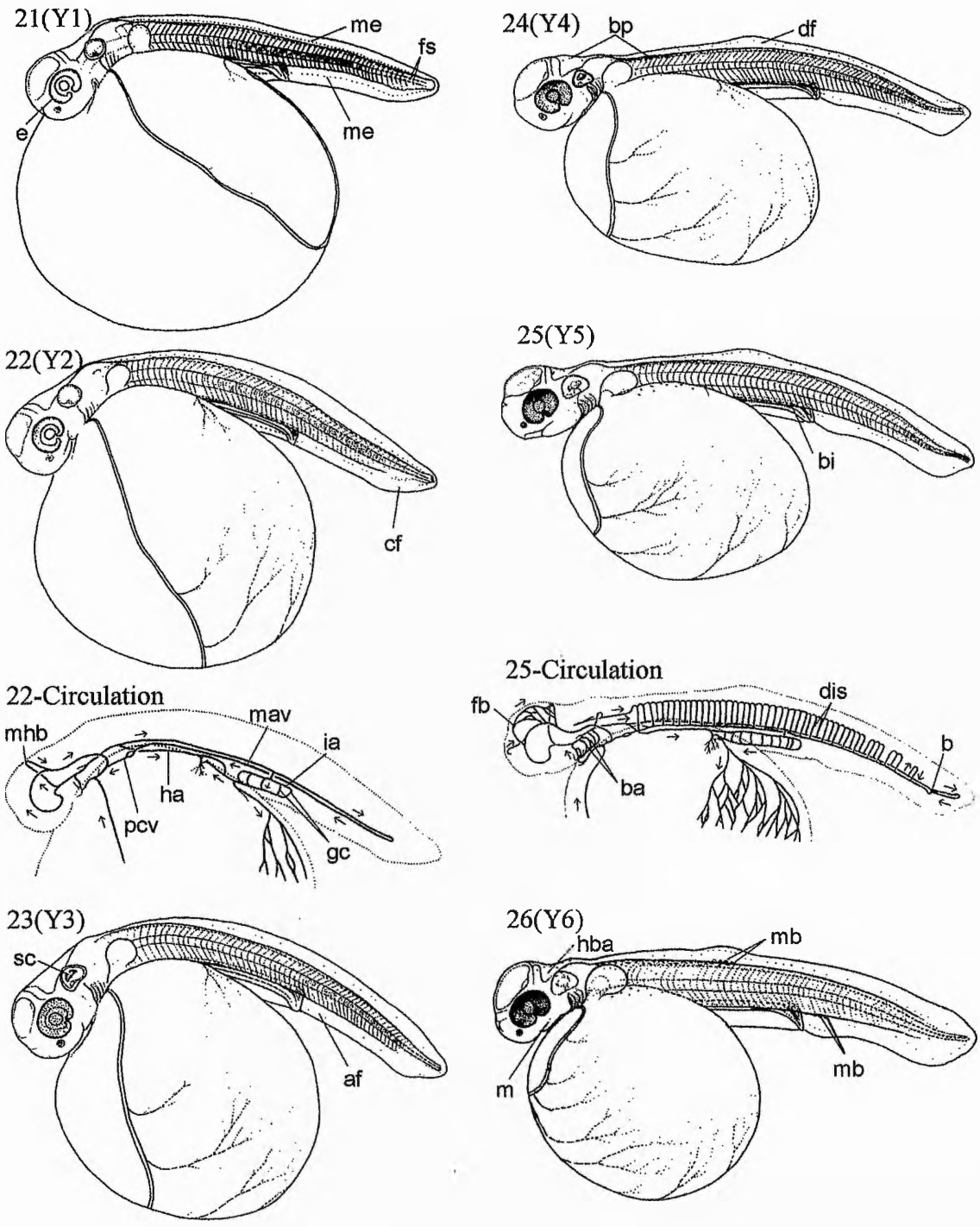



**Fig. 2.3.** Yolksac vascularisation (Y) phase : Steps 21 - 26. Drawings represent embryos at the end of each step. af = future anal fin; b = bypass from caudal artery to caudal vein; ba = branchial arches; bi = bile; bp = body pigmentation; cf = future caudal fin; df = future dorsal fin; dis = intersegmental blood vessels; e = eye pigment; fb = forebrain circulation; fs = false somites; gc = gut capillaries; ha = hepatic artery; hba = hindbrain angle; ia = intestinal artery; m = mouth; mav = median axial vein; mb = muscular buds; me = mesenchyme; mhb = midbrain-hindbrain boundary; pcv = posterior cardinal veins; sc = semicircular canals.

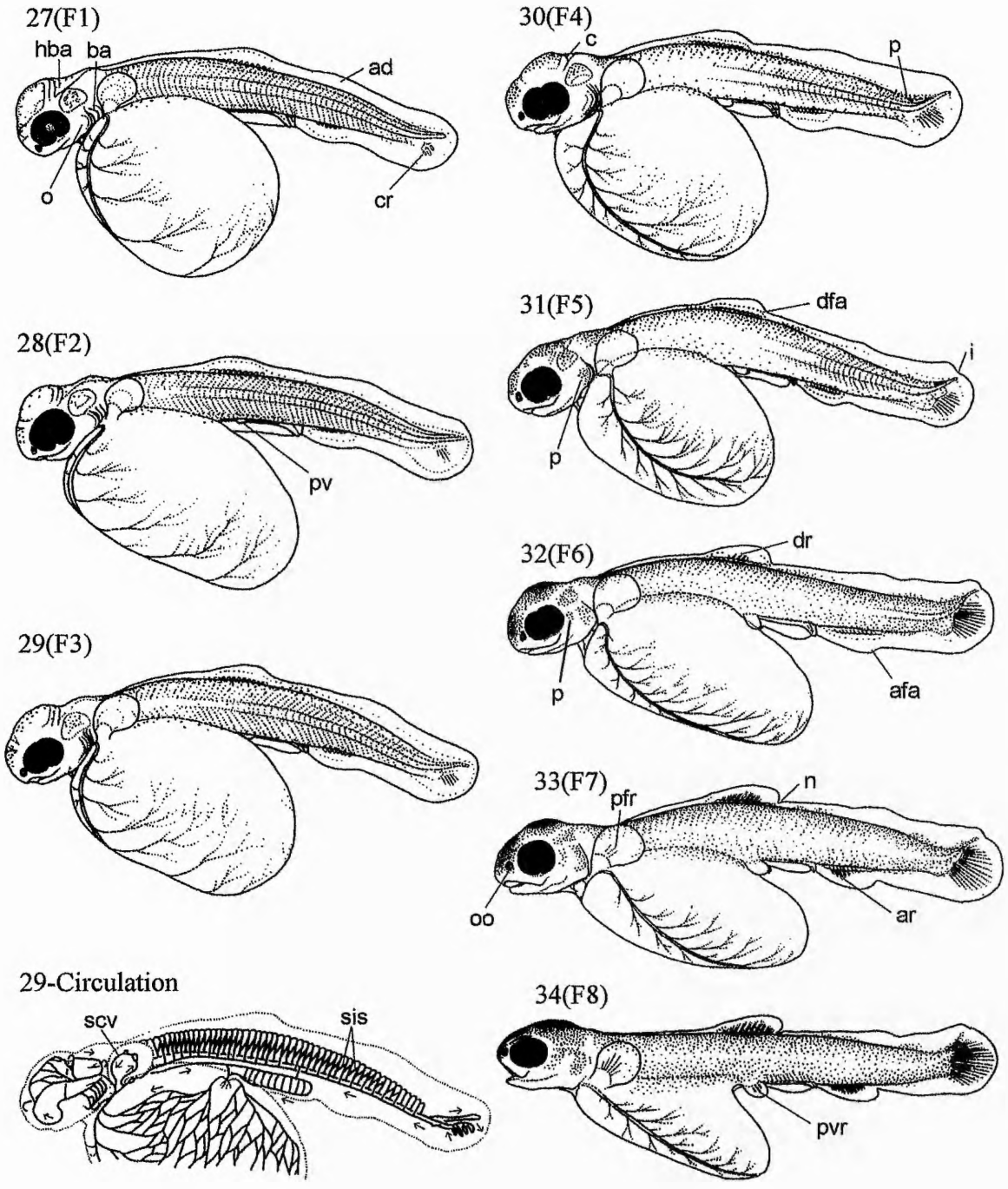



**Fig. 2.4.** Caudal fin ray formation (F) phase : Steps 27 - 34. Drawings represent embryos at the end of each step. ad = future adipose fin; afa = angle at back of anal fin; ar = anal fin rays; ba = branchial arches; c = cerebellum; cr = caudal fin rays; dfa = angle at back of dorsal fin; dr = dorsal fin rays; hba = hindbrain angle; i = indentation in caudal fin; n = notch in dorsal finfold; o = operculum; oo = olfactory organ; p = pigmentation; pfr = pectoral fin rays; pv = pelvic fins; pvr = pelvic fin rays; scv = subclavian vein; sis = superficial intersegmental blood vessels.

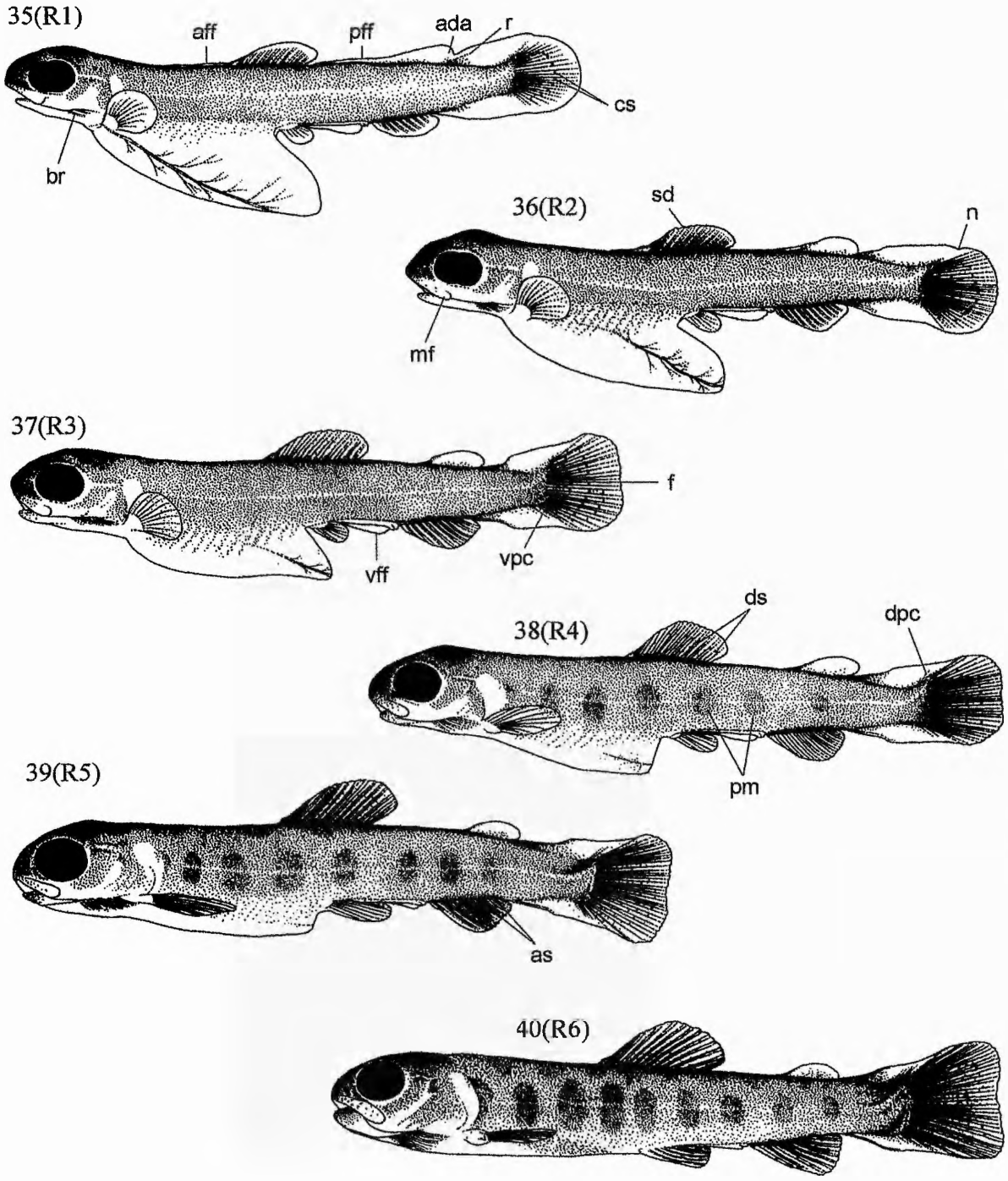



**Fig. 2.5.** Finfold resorption (R) phase : Steps 35 - 40. Drawings represent alevins at the end of each step. ada = angle at back of adipose fin; aff = anterior dorsal finfold; as = anal fin ray segments; br = branchiostegal rays; cs = caudal fin ray segments; dpc = dorsal procurrent caudal fin rays; ds = dorsal fin ray segments; f = developing fork in caudal fin; m = maxillary flap; n = notch in caudal fin; pm = parr marks; pff = posterior dorsal finfold; r = ridge; sd = serration of the dorsal fin; vff = ventral finfold; vpc = ventral procurrent caudal fin rays.

***Developmental Scoring System***

Versions of the scoring system applicable to live and to fixed embryos are presented in Tables 2.1 - 2.4. To calculate the score for an individual embryo:

1) Identify the most advanced 'landmark feature' (bold print) which is present in the embryo. Each landmark has two numbers associated with it. The first number is the individual score for that feature; the second number, in bold print, is the base level score for that landmark.

2) Examine the embryo for each of the features listed between the chosen landmark and the next landmark. For each feature which is deemed to be present, add that feature's individual score to the base level score for the chosen landmark.

3) If the base level score is accompanied by a "+...", examine the embryo for the meristic feature(s) listed in the adjacent columns, and add extra points accordingly.

If the embryo appears to lie at a level of development very close to the chosen landmark, or if the degree of heterochrony of development between embryos is unusually high, it may be advisable to move back to the previous landmark in the scoring system, and begin calculating the score from there. The originally chosen landmark should then be treated as just another feature, and its individual score used rather than its base level score.

**Table 2.1.** The developmental scoring system. Part I - Cleavage egg, Gastrula and Somitogenesis phases.

| Feature | Points (Live) | Meristic Characters | Points (Fixed) |
|---|---|---|---|
| Blastodisc | 1 | | 1 |
| Blastodisc >1/5 high as wide | 2 | | 2 |
| 2 cells | 2.5 | | 2.5 |
| 4 cells[a] | 2.5 | | 2.5 |
| 8 cells[a] | 2.5 | | 2.5 |
| 16 cells[a] | 2.5 | | 2.5 |
| **32 cells or more** | (10) | | (10) |
| **(Blastodisc 3 - 9 cells tall)** | **23** | | **23** |
| Blastodisc 10 - 15 cells tall | 16 | | 16 |
| Individual cells no longer distinguishable | 8 | | 8 |
| Rim of oil droplets no longer elevated | 8 | | 8 |
| Edges of blastodisc contiguous with yolk | 16 | | 16 |
| **Germ ring** | (16) **87** | | (16) **87** |
| Embryonic shield | 8 | | 8 |
| Embryonic shield extends onto 'imprint' | 8 | | 8 |
| Neural plate | 4 | | 4 |
| Distinct head | 4 | | 4 |
| **First brain differentiation** | (4) **116+...** | **Somites**[b] | (4) **116+...** |
| | | 1 point each | |
| | | (Or **111** + **2** points per somite) | |
| Primary neurulation | 4 | . | 4 |
| Otic placodes | 4 | . | 4 |
| Otic placodes begin to develop lumina | 2 | . | 2 |
| Tissue accumulates at future pericardial chamber and pronephri | 2 | . | 2 |
| Optic cup formation begins | 2 | . | 2 |
| Neuromeres | 2 | . | 2 |
| Distinct pronephri | 2 | . | 2 |
| Optic cup and lens outline complete | 2 | . | 2 |
| **Chevron-shaped somites** | (2) **133+...** | Somites | (2) **133+...** |
| | | 1 point each | |

| **Chevron-shaped somites** | (2) **133+...** | Somites<br>1 point each | (2) **133+...** |
|---|---|---|---|
| Choroid fissure formation begins | 2 | . | 2 |
| Stomach | 2 | . | 2 |
| Differentiation of heart into atrium and ventricle | 2 | . | 2 |
| > 1/2 of tailbud free from yolk | 2 | . | 2 |
| Heart bends anteriorly | 2 | . | 2 |
| Pectoral fins | 2 | . | 2 |
| Lens separating from optic cup | 2 | . | 2 |
| **Finfold** | (2) **150+...** | Somites<br>1 point each | (2) **150+...** |
| Somite outgrowths at pectoral fins | 2 | . | 2 |
| 2nd branchial arch | 2 | . | 2 |
| Pronephric ducts turn ventrally at future anus | 2 | . | 2 |
| Olfactory placodes | 4 | . | 4 |
| 3rd branchial arch | 4 | . | 4 |
| Otolith nuclei | 4 | . | 4 |
| Urinary bladder | 4 | . | 4 |
| **First eye pigmentation** | **Stop counting somites**<br>Yolksac vascularisation phase (See Table 2.2) | | |

[a] If the blastodisc consists of an intermediate number of cells (i.e. it is midway through a wave of cleavage divisions), round the number up to achieve the next highest score. [b] As the number of somites is quite an accurate indicator of development during this phase, a simplified version of scoring may be used to save time; no other features are used, and the score is simply 111 + (Somite number x 2).

**Table 2.2.** The developmental scoring system. Part II - Yolksac vascularisation phase.

| Feature | Points (Live) | Points (Fixed) |
|---|---|---|
| **First eye pigmentation** | (2) **236** | (4) **238** |
| Blood flow in hepatic artery | 2 | |
| Mesenchyme ventral to post-anal somites | 2 | 4 |
| Pectoral fins as tall as wide | 2 | |
| Circulation at midbrain- hindbrain boundary | 2 | |
| Ventral mesenchyme thickens at future caudal fin (CF) | 2 | 3 |
| Mesenchyme dorsal to somites | 2 | 3 |
| Head free from yolk as far back as upper jaw | 2 | 2 |
| Pigment > 1/2 way around rim of eye | 3 | 4 |
| Blood turning pink | 3 | |
| Pectoral fins wider at middle than at base | 2 | 4 |
| **Circulation in median axial vein, and capillaries crossing gut** | (4) **262** | |
| **Ventral finfold (VFF) is deeper at future CF** | 4 | (6) **264** |
| Head free from yolk as far back as lower jaw | 4 | 5 |
| Tailbud differentiated to tip | 4 | 5 |
| Rim of pigment around eye reaches to choroid fissure | 4 | 4 |
| Pectoral fins inclining posteriorly | 4 | 4 |
| **Semicircular canals (ventral)** | (3) **284** | (8) **289** |

| | | |
|---|---|---|
| **Semicircular canals (ventral)** | (3) **284** | (8) **289** |
| Semicircular canals (dorsal) | 3 | 8 |
| Circulation at branchial arch 1 | 3 | |
| Circulation at branchial arch 2 | 3 | |
| Deep intersegmental blood vessels | 3 | |
| Caudal artery and notochord vacuolisation to last somite | 4 | 4 |
| Mesenchyme denser and deeper at future anal fin (AF) | 4 | 4 |
| Pigmentation along tops of anterior-most somites | 5 | 5 |
| Pigment on yolk sac | 5 | 5 |
| VFF deeper at future AF | 4 | 4 |
| **Mesenchyme denser and deeper at future dorsal fin (DF)** | 5 | (5) **324** |
| Dorsal finfold (DFF) taller at future DF | 4 | 4 |
| **Circulation over forebrain** | (5) **333** | |
| Circulation at branchial arch 3 | 4 | |
| Circulation at branchial arch 4 | 4 | |
| Pigmentation posterior to front of DF | 5 | 12 |
| Notochord vacuolised to tip | 5 | 12 |
| Muscular buds in AF | 5 | 6 |
| Muscular buds in DF | 5 | 6 |
| Caudal artery produces loop in hypural region of CF | 5 | |
| Mouth open | 4 | 6 |
| Pigment over otic vesicle | 5 | 6 |
| **Caudal fin rays** | Caudal Fin Ray Phase (see Table 2.3) | |

**Table 2.3.** The developmental scoring system. Part III. Caudal fin ray formation phase.

| Feature | Points (Live) | Meristic Characters | Points (Fixed) |
|---|---|---|---|
| **Caudal fin rays** | **375+...** | **CF Rays** | **375+...** |
| | | 5 points each | |
| Hindbrain angle <60° | 2 | . | 2 |
| Pigment over forebrain | 2 | . | 2 |
| Superficial intersegmental blood vessels | 2 | . | |
| Hindbrain angle <45° | 2 | . | 4 |
| DFF taller at future adipose fin | 4 | . | 4 |
| >6 DF muscular buds | 3 | . | 3 |
| Pigmentation posterior to back of DF | 3 | . | 3 |
| **Pelvic fin rudiments** | (4) **398+...** | 5 points per CF ray | (4) **398+...** |
| >8 DF muscular buds | 4 | . | 4 |
| Pigment along ventral edges of somites | 4 | . | 4 |
| Hindbrain angle <30° | 4 | . | 4 |
| Circulation among caudal fin rays | 4 | . | |
| Filaments forming on branchial arch 1 | 4 | . | 9 |
| Operculum covers all of 2nd branchial arch | 3 | . | |
| DF inclining posteriorly | 3 | . | |
| Pigment on DFF | 3 | . | 8 |
| **Pigment at most posterior somite** | (4) **430+...** | 5 points per CF ray | (6) **432+...** |
| Pelvic fins >1/2 depth of VFF | 4 | . | 5 |
| Capillaries in filaments of branchial arches 1 & 2 | 4 | . | |
| Filaments forming on branchial arch 3 | 4 | . | 6 |
| Cerebellum lies flat against rest of hindbrain | 4 | . | 6 |
| AF inclining posteriorly | 4 | . | |
| Indentation in CF opposite tip of notochord | 3 | . | 8 |
| 'Step' in median axial vein | 3 | . | |
| Capillaries in filaments of branchial arch 3 | 4 | . | 7 |
| Filaments forming on branchial arch 4 | 4 | . | |
| Operculum covers all of branchial arch 3 | 3 | . | |
| Pigment on operculum | 3 | . | 7 |
| **Rudiments of dorsal fin rays** | (4) **475+...** | 5 points per CF ray | (6) **477+...** |

| | | | |
|---|---|---|---|
| **Rudiments of dorsal fin rays** | (4) **475+...** | 5 points per CF ray | (6) **477+...** |
| Angle at back of DF >60° | 3 | . | |
| Tips of pelvic fins level with VFF | 4 | . | 5 |
| Pelvic fins extend past VFF | 3 | . | 5 |
| Capillaries in filaments of branchial arch 4 | 4 | . | |
| Pigment between back of eye and operculum | 4 | . | 5 |
| **Tip of caudal artery unites with CF plexus** | (4) **496+...** | 5 points per CF ray | |
| Angle at back of DF >90° | 3 | . | |
| Notch in DFF behind DF | 4 | . | 5 |
| Inner projection in olfactory cavity | 3 | . | 5 |
| >4 dorsal fin rays | 3 | . | 4 |
| Pectoral fin rays | 3 | . | 4 |
| **Anal fin rays** | 3 | 5 points per CF ray | (4) **514+...** |
| Pelvic fins wider at middle than at bases | 5 | . | 5 |
| >6 dorsal fin rays | 6 | . | 6 |
| >3 anal fin rays | 6 | . | 6 |
| **Pelvic fin rays** | (5) **536+...** | 5 points per CF ray | 10 |
| Angle at back of AF >90° | 6 | . | |
| >6 anal fin rays | 6 | . | 9 |
| >8 dorsal fin rays | 6 | . | 9 |
| Angle at back of DF >120° | 6 | . | |
| **Segments in caudal fin rays** | Finfold Resorption Phase (See Table 2.4) | | |

**Table 2.4.** The developmental scoring system. Part IV. Finfold resorption phase.

| Feature | Points (Live) | Meristic Characters | | | Points (Fixed) |
|---|---|---|---|---|---|
| **Segments in caudal fin rays** | **559+...** | CF Rays 5 points each | **Segments in CF Rays** 1 point each | | **559+...** |
| >8 anal fin rays | 5 | . | . | | 7 |
| Angle at back of adipose fin >60° | 5 | . | . | | |
| Ridge developing behind adipose fin | 5 | . | . | | 7 |
| **DF becomes serrated** | (4) **657+...** | **Stop counting CF Rays** | Segments in CF Rays 1 point each | **Branchiostegal Rays** 6 points each | (4) **657+...** |
| Angle at back of AF >120° | 3 | | . | . | |
| >1/2 of anterior DFF resorbed | 3 | | . | . | 5 |
| Posterior DFF resorbed past back of DF | 4 | | . | . | 4 |
| Ridge developing behind AF | 3 | | . | . | 4 |
| CF equally tall in front of and behind notch | 4 | | . | . | 4 |
| >3/4 of anterior DFF resorbed | 4 | | . | . | 4 |
| >1/2 of posterior DFF resorbed | 4 | | . | . | 4 |
| DFF behind adipose fin fully resorbed | 4 | | . | . | 4 |
| Maxillary flap protrudes posteriorly | 3 | | . | . | 3 |
| **Anterior DFF fully resorbed** | (5) **688+...** | **V.P.C. Fin Rays**[a] 8 points each | Segments in CF Rays 1 point each | Branchiostegal Rays 6 points each | (6) **689+...** |

| **Anterior DFF fully resorbed** | (5) **688+...** | **V.P.C. Fin Rays**[a] 8 points each | Segments in CF Rays 1 point each | Branchiostegal Rays 6 points each | (6) **689+...** |
|---|---|---|---|---|---|
| Posterior DFF fully resorbed | 5 | . | . | . | 6 |
| Angle at back of adipose fin >90° | 5 | . | . | . | |
| CF taller behind than in front of notch | 5 | . | . | . | 7 |
| Finfold over ridge behind AF resorbed | 5 | . | . | . | 8 |
| Angle at back of adipose fin >120° | 5 | . | . | . | |
| >5 pelvic fin rays | 5 | . | . | . | 7 |
| Parr marks | 10 | . | . | . | 10 |
| 1st row of segments in DF rays | 9 | . | . | . | 9 |
| Post-anal parr marks | 9 | . | . | . | 9 |
| **1st row of segments in anal fin rays** | (8) **754+...** | V.P.C. Fin Rays 8 points each | Segments in CF Rays 1 point each | Branchiostegal Rays 6 points each | (8) **754+...** |
| VFF resorbed >1/2 way along length of pelvic fins | 6 | . | . | . | 6 |
| >8 segments in 1st row in DF rays | 8 | . | . | . | 8 |
| >6 segments in 1st row of AF rays | 8 | . | . | . | 8 |
| 2nd row of segments in DF rays | 8 | . | . | . | 8 |
| >8 segments in 1st row of AF rays | 9 | . | . | . | 9 |
| VFF resorbed past backs of pelvic fins | 9 | . | . | . | 9 |
| 2nd row of segments in AF rays | 8 | . | . | . | 8 |
| VFF shallower than protruding anus | 8 | . | . | . | 8 |
| >6 segments in 2nd row in DF rays | 6 | . | . | . | 6 |
| 3rd row of segments in DF rays | 6 | . | . | . | 6 |
| VFF fully resorbed | 6 | . | . | . | 6 |

[a] V.P.C. fin ray = Ventral procurrent caudal fin ray

***Development after first feeding***

Branchiostegal rays are produced at the rate of one every 6.9 days at 6°C. Ray formation continues until approximately 7 days after first feeding (mean fish standard length (S.L.) = 22.8 ± 0.38mm S.E.), after which time the number of branchiostegal rays stabilises at 10.8 ± 0.4.

Dorsal and ventral procurrent caudal fin rays are produced at the rate of one every 5.5 days and one every 7.5 days respectively (Fig. 2.6a). Their formation continues at a regular rate until approximately 50 days after first feeding (mean fish S.L. = 31.2 ± 1.1mm), after which it slows and then stops. The final number of rays produced is 13.3 ± 0.6 (dorsal) / 11.5 ± 0.7 (ventral).

A new row of segments in the caudal fin is added approximately every 15.5 days (Fig. 2.6b). When sampling was ended, 80 days after first feeding (mean fish S.L. = 45.6 ± 3.7mm), the rate of formation of new rows was undiminished; their number was, however, becoming increasingly difficult to determine due to overlap in position between some regions of successive rows. The same was true of rows of segments in the anal fin, which are produced at the rate of one every 17.4 days.

The rates given above are for the total period of study, both before and after first feeding. The rates of dorsal and ventral procurrent ray formation both increased after provision of food and transfer from 6°C to 13°C, from 0.14 rays $day^{-1}$ to 0.18 $rays.day^{-1}$ and from 0.13 $rays.day^{-1}$ to 0.15 $rays.day^{-1}$ respectively. Production of rows of segments in the fins actually slowed, from 0.080 $rows.day^{-1}$ to 0.061 $rows.day^{-1}$ in the caudal fin, and from 0.077 $rows.day^{-1}$ to 0.054 $rows.day^{-1}$ in the anal fin.

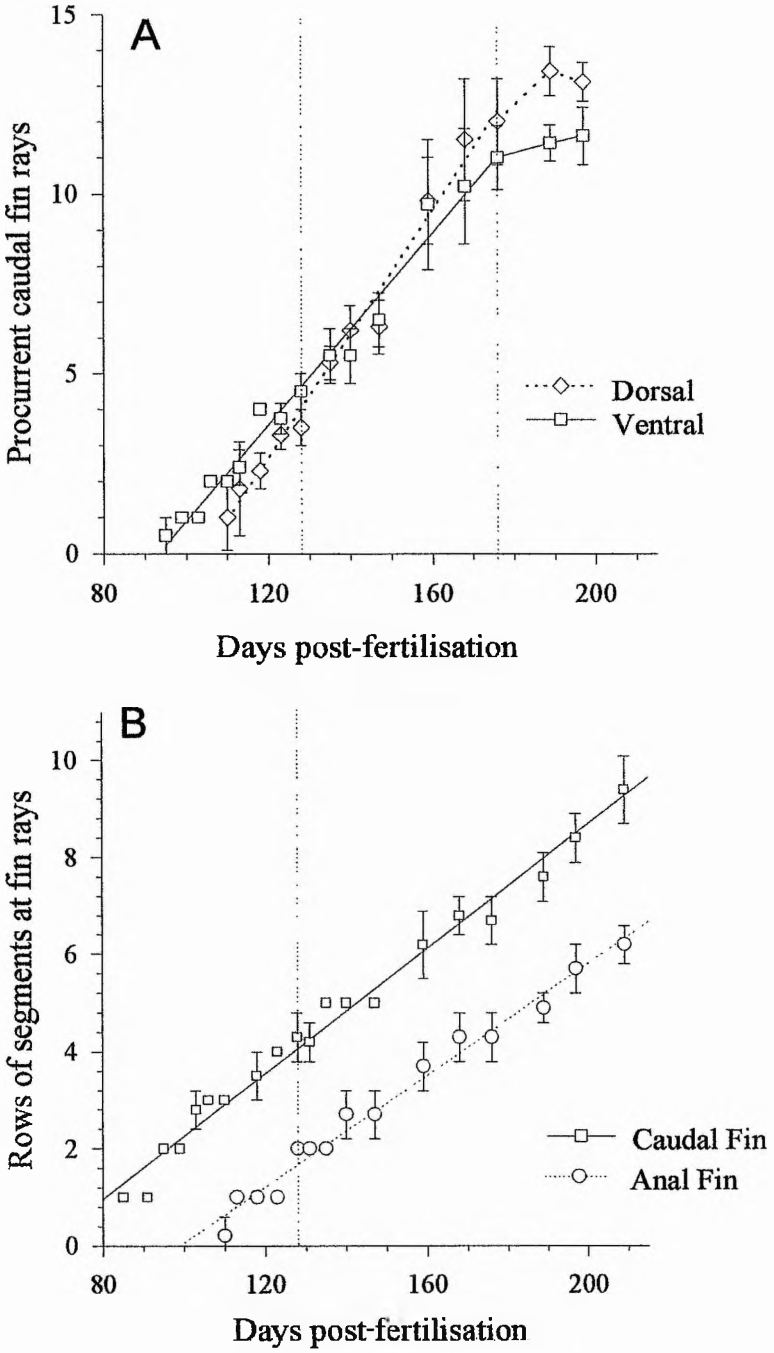



**Fig. 2.6.** Increases in certain meristic characters from the finfold resorption phase into the period of initial feeding. The first vertical dotted line in each graph indicates the time of first feeding. Data from 1996 fertilisation. Points represent mean ± S.E. **A**: Dorsal and ventral procurrent caudal fin rays. The second vertical line indicates the estimated end of regular fin ray formation. **B**: Rows of segments at the fin rays in the caudal and anal fins.

***Effects of temperature and maternal migratory type on development***

The scoring system (Tables 2.1 - 2.4) was designed based on development at 6°C, but the score increased linearly with age at all temperatures studied. The rate of increase in score was 12.5 points.day$^{-1}$ at 10°C, 7.9 points.day$^{-1}$ at 6°C, and 3.8 points.day$^{-1}$ at 2°C (Fig. 2.7, based on fixed specimens). P-values were less than 0.001 for least-squares regressions at all three temperatures. The effect of temperature on the rate of development is further illustrated in Fig. 2.8.

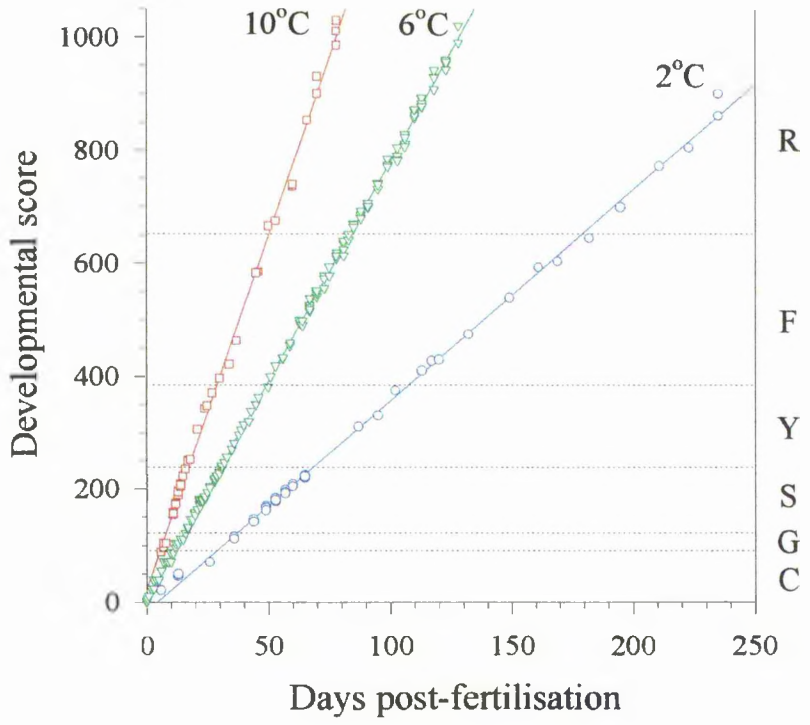



**Fig. 2.7.** The relationship between developmental score and age at different temperatures. Data from 1996 fertilisation. The horizontal lines indicate the boundaries of successive phases: Cleavage egg (C), Gastrula (G), Somitogenesis (S), Yolksac vascularisation (Y), Caudal fin rays (F), Finfold resorption (R).

**Fig. 2.8** Three trout of the same age (200 days post fertilisation) but reared at different temperatures: 10°C (top), 6°C (middle) and 2°C (bottom). Scale bar = 1cm.

Temperature altered the relative timing of certain developmental changes, which in general were delayed at lower temperatures. The following findings are for embryos from the 1996 fertilisation unless stated otherwise:

*Heartbeat*: At 10°C, all embryos with a score of 183 or greater had beating hearts. At 2°C, heartbeat was observed in some embryos of score 183 - 185, while others with scores as high as 197 displayed no heart movements.

*Trunk muscle contractions*: At 10°C, all embryos with a score of 197 or more exhibited spontaneous contractions in the anterior somites. These movements did not begin until after scores of at least 203 at 6°C, and after scores of 211 at 2°C.

*Bile production*: At 10°C and 6°C, at least some embryos had begun to produce intestinal bile by scores of approximately 350. At 2°C, no embryos with scores of 358 or less had begun such production.

*Movement of pectoral fins:* The first signs of movement in the pectoral fins were seen in an embryo of score 340 at 10°C. However, there was no such movement in embryos as late as score 381 at 6°C. At both the higher temperatures, all embryos exhibited twitches of pectoral fins by scores of approximately 400. Fin movement at 2°C did not begin until after scores of 415.

*Spontaneous hatching*: At 10°C and 6°C, embryos from the 1996 fertilisation were observed to hatch as early as score 542 (10°C) / 555 (6°C). At 2°C, no embryos with scores less than 597 hatched spontaneously. At 6°C, all embryos with scores of 613 or greater had hatched. At 10°C and 2°C, complete hatching did not occur until scores of 665 (10°C) / 671 (2°C). The effect of temperature on the timing of hatch was even greater for the 1995 fertilisation; spontaneous hatching began at an estimated score of 561 at 10°C, but did not begin until an estimated score of 632 at 2°C.

Rearing temperature, but not maternal migratory type, significantly affected the final number of somites produced ($P<0.01$) (Table 2.5). Tukey multiple comparison tests found mean values of final somite number to be significantly reduced at 10°C, relative to both 2°C ($P<0.001$) and 6°C ($P=0.026$).

Although very small differences in patterns of development were found between different maternal groups, there were no consistent differences in the timing of appearance of any feature studied with maternal migratory type, based on the 1995 fertilisation. Rates of somite formation were not significantly affected by the migratory type of the female parent at any temperature (Fig. 2.9, Table 2.5).

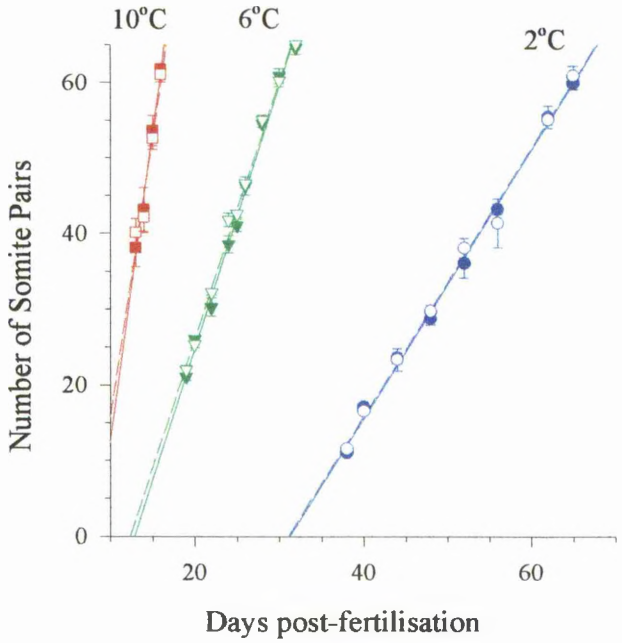



**Fig. 2.9.** Increase in number of somite pairs with age at different temperatures. Data from 1995 fertilisation. Closed symbols and unbroken lines represent offspring of anadromous females, open symbols and broken lines the offspring of freshwater resident females.

**Table 2.5.** Effects of temperature and maternal migratory type on rate of somite formation and final number of somites produced. Data from 1995 fertilisation. Counts of final somite numbers were taken from embryos in steps 21 (Y1) to step 23 (Y3), and are presented as means (± S.E.); n = 20. A = Offspring of anadromous females; R = Offspring of freshwater resident females.

| | 10°C | | 6°C | | 2°C | |
|---|---|---|---|---|---|---|
| | A | R | A | R | A | R |
| Mean daily rate of somite formation | 8.13 | 7.35 | 3.47 | 3.41 | 1.77 | 1.77 |
| | 7.74 | | 3.44 | | 1.77 | |
| Mean final somite number | 63.1 (1.3) | 62.4 (1.5) | 63.5 (1.2) | 63.3 (1.3) | 63.7 (0.8) | 63.7 (0.9) |
| | 62.7 (1.5) | | 63.4 (1.2) | | 63.7 (0.9) | |

## Discussion

### *Developmental steps and scoring system*

The format of the developmental steps produced in this study is modelled on that used by Balon (1980) for the ontogeny of the lake charr (*Salvelinus namaycush* L.). He divided the entire period from fertilisation to the end of yolk utilisation into twelve steps, with successive steps separated by 'thresholds'; each threshold is a combination of abrupt functional changes marking a distinct ontogenetic 'leap', according to the theory of saltatory development. In this study, boundaries between steps were set at points in development that could be recognised with relative ease; they are not intended to indicate times of particular developmental significance. This allows a greater number of steps to be described, enabling more precise estimation of the level of development of individual embryos.

Steps have certain advantages over the more discrete 'stages' used by earlier authors (e.g. Ballard 1973c; Vernier 1969). Such stages, each describing the embryo at a particular instant during ontogeny, take no account of intermediate embryos which may fall between successive stages. Also, as steps are based on a number of features rather than just one, their use reduces the impact on embryo classification of minor heterochrony in developmental patterns between individuals. This also distinguishes steps from the 'states' of Gorodilov (1996), the latter being defined by a single feature.

Because of its flexible nature, the scoring system developed in this study is also buffered against the slight shifts in developmental patterns that arise between embryos, even those from the same family reared under identical conditions (Armstrong & Child 1965). But because it is more precise than the series of steps, use of the scoring system highlights the differences in overall developmental rate that also arise. For example, around the time of first feeding, a single sample of six free embryos from one maternal group at one temperature can include specimens differing by up to 30 points in score, yet all belonging to Step 40 (R6). Application of the scoring system will enable far more accurate analysis of development in trout embryos in future studies, such as for comparisons between families of different egg size, or to

quantify relationships between embryo size and level of development under different conditions (see Chapter 3). Similar scoring systems may be prepared for other species, given a suitably precise and detailed description of that species' embryonic development.

Gorodilov (1989) reported practically identical development in trout and Atlantic salmon. However, the present study indicates significant variation in development between these two species. For example, pectoral fins are present by the end of Step 17 (S5) in trout, before the 39 somite stage; but do not appear until the 56 somite stage in Atlantic salmon (Gorodilov 1989), equivalent to Step 20 (S8). Body pigmentation develops during Step 24 (Y4) in trout, but not until the equivalent of Step 27 (F1) in salmon - a difference in score of approximately 65 points. Differences in the timing of appearance of the finfold, notochord vacuolisation, gill filaments, and rows of segments in the fins are also present between the two species.

***Development after first feeding***

Although previous studies on salmonid development have extended only to the times of first feeding and yolk exhaustion (e.g. Heming 1982; Gorodilov 1996), certain developmental processes that begin during the finfold resorption phase extend well into the feeding phase. The numbers of dorsal and ventral procurrent caudal fin rays may prove useful as a simple indicator of development for the first one to two months of this phase. Rows of segments in caudal and anal fin rays could also be used as developmental indicators; although they are formed more slowly than procurrent fin rays, and would therefore be less precise, their production continues for longer. Counts of branchiostegal rays would be of little use as such an indicator, given the short length of time for which they are produced after first feeding.

The rate of formation of such structures might have been expected to show a greater increase after first feeding, given the 7°C rise in water temperature after transfer to new tanks. However, the rate of formation of rows of fin ray segments was actually reduced. This is consistent with reports in other species that the effect of

temperature on developmental rate is greatly reduced as development progresses (Collins & Nelson 1993; Hunt von Herbing *et al.* 1996).

***Effects of temperature and maternal migratory type on development***

The linear increase in developmental score in trout incubated at 2°C, 6°C and 10°C indicates that the relative durations of successive subdivisions of development are maintained over this temperature range. This is in agreement with findings for other salmonid species (Kamler & Kato 1983; Gorodilov 1989). Gorodilov (1992) suggested that the timing of embryonic development may be under the control of a small number of endogenous pacemakers, with the same pacemaker controlling cleavage divisions, somite formation and possibly other aspects of early ontogeny.

Temperature-dependent shifts in relative timing were observed in the development of function of certain systems, and also in the onset of hatching; but the relative timing of formation of structural elements was unaffected. This is in agreement with the conclusions of Garside (1959), Vernier (1969) and Gorodilov (1983), although they reported changes in the timing of bile production and hatching only; effects of temperature on first muscle contractions and fin movements were either not studied or no effects were found. Delayed onset of heartbeat at low temperatures has also been reported by Pavlov (1984), although he also reported retardation of pelvic fin development under such conditions, which was not observed in this study.

Whether the earlier onset of these physiological changes at higher temperatures is advantageous to the embryo remains unknown. It has been suggested that movements of the embryo within the chorion, particularly movements of the pectoral fins, serve to improve circulation of the perivitelline fluid and thus improve uptake of oxygen by the embryo (Peterson & Martin-Robichaud 1983). As the oxygen requirements of embryos are greater at higher temperatures (Rombough 1987), and dissolved oxygen concentrations in the water will be lower, the earlier onset of such movements may be necessary, in spite of the presumed increase in energy expenditure.

The possibility of oxygen deficit at high temperatures may also explain the earlier onset of hatch. Critical oxygen levels increase continually while the embryo develops inside the egg, peaking just before hatching; but emergence from the chorion is associated with a subsequent sharp decrease in the critical oxygen level for survival of the embryo (Kamler & Kato 1983; Rombough 1987), suggesting that the chorion limits the rate of oxygen diffusion into the egg. Thus the embryo may need to emerge from the egg relatively earlier to ensure an adequate oxygen supply, when environmental temperature has been continuously high (Heming 1982; Pavlov 1984; Rombough 1987) or has just risen sharply during a critical period towards the end of embryonic development (Wells & Pinder 1996). The higher frequency of trunk movements at higher temperatures may aid the hatching process, both by physically tearing the chorion and by distributing the hatching enzymes inside the egg. This dependence of the time of hatching on environmental conditions makes its use as a staging device potentially unreliable. A more accurate assessment of the level of developmental advancement, based on features other than emergence from the chorion, should be made prior to such comparisons, using either the series of steps outlined above or the scoring system.

Heterochrony during embryonic development has been observed between closely related species with different lifestyles (Balon 1981), and has been put forward as a possible source of evolutionary change (Freeman 1982). However, it seems more plausible that such change would arise due to differences in the timing of structural changes, such as those described by Johnston (1993), than by the changes in the timing of onset of physiological functions due to temperature found in this study.

The lack of differences in developmental patterns between offspring of migratory and of non-migratory females in this study contrasts with the findings of Halacka (1995). He reported that organs appeared later, relative to somite number, in offspring of freshwater resident trout, when compared to the findings of Pavlov (1989) for offspring of anadromous trout. Pavlov's study was carried out at a higher ambient temperature - 4.8°C compared to the mean of 1.6°C used by Halacka (1995). However, given the lack of effects of temperature and maternal migratory type on organ formation in this study, such divergent findings may reflect differences in

parental stock between the studies; populations from different watercourses usually show strongly significant genetic differences (Hindar *et al.* 1991).

The reduction at 10°C in the final number of somites produced is consistent with the findings of Pavlov (1984), and those of Kwain (1975) and Beacham & Murray (1986) for vertebral number. In sticklebacks, lower numbers of vertebrae, usually correlated with final somite number, have been found to be associated with reduced predation in laboratory experiments (Swain & Lindsey 1984). If a similar situation occurs in trout, then fish reared at temperatures near the upper end of the range for normal development may be at a disadvantage.

In conclusion, the series of developmental steps, and the developmental scoring system, provide two new methods for quantifying the developmental advancement of embryos of *S. trutta*, based specifically on findings for that species. The steps provide a more complete description of early ontogeny in trout than can be found in previous publications, and have advantages over the staging systems commonly used in studies of salmonid development. The scoring system provides greater precision, sufficient to quantify within-sample variation in levels of developmental advancement. Both methods avoid use of those changes in organ function, and events such as hatching, whose relative timing is affected by water temperature. Maternal migratory type was not found to affect early patterns of development. These two new methods make possible more accurate examination of the relationships between development and various aspects of embryonic growth, such as are presented in Chapters 3 and 5.

# Chapter 3: Temperature, egg size and the utilisation of yolk for growth and development in trout

## Introduction

The ability of teleost embryos to begin feeding exogenously after hatching depends to a large extent on the size and degree of development which they achieve from their yolk supply (Miller *et al.* 1988; Marteinsdottir & Steinarsson 1998). Size at first feeding, which can also affect parameters such as swimming endurance (Ojanguren *et al.* 1996) and the establishment and defence of territories (Chapman 1962), is known to vary with egg size (Beacham & Murray 1985). Within a species, the mean size of the unfertilised eggs, and thus the quantity of yolk supplied to the embryo, can vary substantially between stocks (Blaxter & Hempel 1963; Beacham & Murray 1987; Tallman & Healey 1991), between females within a stock (Kazakov 1981; Beacham & Murray 1987), and within females (Blaxter & Hempel 1963). Egg size usually increases with increasing maternal size and condition (Blaxter & Hempel 1963; Kazakov 1981; Thorpe *et al.* 1984; Chambers *et al.* 1989; Jonsson *et al.* 1996). As well as the variation with egg size in the total amount of yolk available to the growing embryo, variation may also exist in the amount of tissue which the embryo produces from a specific quantity of yolk (Blaxter & Hempel 1966; Kamler & Kato 1983). The value of mass of tissue produced as a proportion of the mass of yolk utilised is frequently referred to as the 'efficiency' of yolk utilisation, and the term 'efficiency' has also been used with reference to the length achieved by an embryo from a given quantity of yolk (e.g. Blaxter & Hempel 1966; Hamor & Garside 1977; Kamler & Kato 1983; Arul 1991; Polo *et al.* 1991; Peterson *et al.* 1995; Overnell 1997)

In addition to the effects of egg size, the size of the embryo produced from a given quantity of yolk is known to vary with environmental temperature in many fish species (Heming 1982; Kamler & Kato 1983; Wang *et al.* 1987; Polo *et al.* 1991). This may be due to an effect of temperature on the relative amount of energy expended on metabolism and on physical activity rather than on growth (Blaxter & Hempel 1966; Hamor & Garside 1979; Rombough 1987; Fukuhara 1990). The

optimum temperature for conversion of yolk into increase in length may differ from that for increase in mass, resulting in variations in the condition factor of the embryo (Beacham & Murray 1985; Morley 1998).

While the utilisation of yolk for growth has been studied in a wide range of species, the use of yolk to achieve advances in developmental state has been less well studied. Pavlov & Moksness (1995) found that the quantity of yolk used by wolffish (*Anarhichas lupus* L.) embryos to reach a given developmental stage decreased with increasing temperature, while Fukuhara (1990) reported that, in the Japanese flounder (*Paralichthys olivaceus* Temminck & Schlegel), temperature affected the amount of yolk remaining at key stages of development such as formation of the pectoral fins and pigmentation of the eyes.

The aim of the study described in this chapter was to examine the gains made by trout embryos and alevins from utilisation of their yolk supplies. Yolk is processed to produce increases in tissue mass, but is also necessary for the embryo to differentiate from a single cell into an independent organism. Using the scoring system described in Chapter 2, the degree of development can be precisely determined, and examined entirely independently of growth. The extent to which temperature and egg size affect the use of yolk for the two separate processes of growth and development has thus been examined.

## Materials and Methods

### *Fish husbandry*

The embryos and alevins examined in this chapter were sampled from six of the ten maternal groups produced from the 1996 fertilisation described in Chapter 2 (see p. 51-52). The eggs from these fish were judged to be representative of the range of egg sizes in all ten maternal groups. The mean fork length of the six relevant female parents was 443 ± 18 mm (S.E.) (see Appendix I). Only two of the female parents obtained in 1996 proved to be anadromous; although their offspring were included in the present study, the effects of maternal migratory type on the phenotype of the offspring have not been examined.

Samples of unfertilised eggs (n = 16) from each female were frozen and stored at -20°C. Each of the six maternal groups was subdivided among egg trays supplied with river water at two temperatures, 10°C (10.0 - 10.4°C) and 2°C (1.6 - 2.4°C). Forty-three days post-fertilisation (d.p.f.), when the embryos reared at 10°C began to hatch spontaneously (step F6), 50 eggs from each maternal group were transferred to egg trays supplied with water at 10°C. The temperature of the water in these trays was then allowed to fall, over a 6-hour period, to 6°C (5.4 - 6.4°C), at which temperature the alevins in this '10 → 6°C' group were subsequently incubated. At 149 d.p.f., the embryos reared at 2°C would hatch when agitated, but not spontaneously; nevertheless, they were considered, based on examination of between two and three embryos from each maternal group, to have attained a degree of development comparable to that at which the 10°C embryos had begun to hatch i.e. step F6. (Note: the scoring system described in Chapter 2 had not been developed at the time of sampling for the experiment detailed in this chapter.) Fifty eggs from each maternal group reared at 2°C were transferred to trays supplied with water at 6°C, the temperature being adjusted over a 6 hour period, to constitute a '2 → 6°C' group. For convenience, the time of transfer at each temperature will henceforth be referred to as the 'hatch' stage. Alevins were reared under the four temperature regimes (constant 10°C, '10→6°C', '2→6°C' and constant 2°C) until such time as they were considered to be near to the point of exhaustion of their yolk supplies.

Embryos (n=8) were sampled from each maternal group at the 'hatch' stage at 10°C and 2°C. Additional samples (n = 4-8 from each maternal group) were collected at 60, 70 and 78 d.p.f. at constant 10°C; at 67, 84 and 104 d.p.f. from the '10→6°C' group; at 182, 191 and 203 d.p.f. from the '2→6°C' group; and at 200, 230 and 235 d.p.f. at constant 2°C. Specimens were killed by overdose of MS-222 (ethyl m-aminobenzoate) anaesthetic, fixed overnight in buffered 10% formalin (pH 7.2) and stored in 0.1% sodium azide in phosphate buffered saline (pH 7.2).

***Examination of specimens***

The unfertilised eggs from each female were thawed and fixed in buffered 10% formalin (pH 7.2) overnight. After removal of the chorion, the yolk from each

egg was blotted dry and the wet weight was measured on a Mettler AE50 balance (Mettler Instruments, High Wycombe). The yolk was then dehydrated in a Christ Alpha 1-4 freeze-drier (Cryotechnics, Edinburgh) for seven days and weighed again. (Dry mass of embryos and of yolk has been shown to be virtually identical between fresh and formalin-fixed specimens (Blaxter & Hempel 1966)). Values of mean dry mass of initial yolk per egg (henceforth referred to as 'egg size') were calculated for each female parent.

Fixed embryos and alevins (henceforth referred to simply as 'alevins') were examined under a dissecting microscope and assigned a developmental score, using the scoring system described in Chapter 2. The yolk was removed, taking care to leave the yolksac membrane attached to the alevin. The alevin was blotted dry and weighed. As many of the less advanced specimens were curved in the dorsoventral plane, lengths of all specimens were measured using a dissecting microscope and JVC TK-1281 colour video camera (JVC, Tokyo) attached to a Video-Plan Image Analysis System (Kontron Electronics, Basel). Alevins which were curved in the lateral plane as a result of fixation were held flat between two glass slides. Standard length was measured from the tip of the snout to the anterior margin of the caudal fin. Alevins and yolksacs were subsequently stored at -20°C before being freeze-dried for seven days and weighed. The mass of yolk utilised by each individual alevin was estimated by subtracting the dry mass of the remaining yolksac from the initial mean egg size for the relevant female parent.

***Statistical analysis***

All statistical analysis was carried out using the Minitab statistical analysis package (Minitab Inc., U.S.A.). Mean values of developmental score, standard length, dry body mass, and dry mass of yolk utilised at the 'hatch' stage at 10°C and 2°C were analysed by two-way GLM (general linear model) ANOVA, using embryonic temperature (two possible values: 10 and 2°C) and egg size (six possible values) as factors. The mean mass of the yolk remaining in alevins in the final samples collected at each temperature was analysed by three-way GLM ANOVA, using embryonic temperature, post-'hatch' temperature and egg size as factors.

The effects of temperature and of egg size on the relationships between developmental score, standard length, dry mass of the alevin and dry mass of yolk utilised were examined by multiple regression analysis. For each relationship between a variable $Y$ and a variable $X$, each observation was assigned a $T$ value for embryonic temperature ($T$ = 0 for fish reared at 2°C as embryos, $T$ = 1 for fish reared at 10°C as embryos), an $AT$ value for the temperature experienced as an alevin i.e. after the 'hatch' stage ($AT$ = 0, 1 or 2 for alevins reared at 2°C, 6°C and 10°C respectively), and an $E$ value ($E$ = the mean egg size for the female parent of that particular specimen). A regression was then constructed for the relationship, including factors such as $X$ and $X^2$; $T$, $AT$ and $E$ to test for possible simple additive effects of embryonic temperature, of post-'hatch' temperature, and of egg size; and factors such as $T$ x $E$, $AT$ x $E$, $T$ x $X$, $E$ x $X$ and $T$ x $AT$, to examine possible interactions between embryonic temperature, post-'hatch' temperature, egg size and the $X$ variable. Factors whose individual significance values exceeded P=0.05 were successively omitted and new regressions constructed, until a regression model was arrived at for which all factors used had individual significance values of P<0.05, following the 'top-down' method described by Zar (1996). The process was repeated replacing the variable $E$ with the variable $G$ ($G$ = 0 - 5 depending on the maternal group), and the goodness of fit of the final regressions produced using the two different variables was compared. From the final regression models chosen, predicted values of developmental score, standard length and body mass after utilisation of 24mg (dry weight) of yolk, and at the point of exhaustion of the yolk supply, were calculated for different temperature regimes and egg sizes.

## Results

### *Unfertilised eggs*

The mean egg size (i.e. dry mass of yolk per unfertilised egg) for each of the six female parents ranged from 24.3 ± 0.3mg to 36.8 ± 0.7mg. Egg size was not significantly correlated with maternal fork length. However, the water content of the eggs, which ranged from 53.4% to 64.9%, was negatively correlated with maternal fork length (Pearson's correlation, $r$ = -0.242, P = 0.02). Larger females thus produced eggs with relatively lower water content, but of similar dry mass, compared

to smaller females. Percentage water content was not significantly correlated with the dry mass of the yolk, indicating that the concentration of endogenous nutrients did not differ between large and small eggs.

***Developmental score and size at the 'hatch' stage***

Assessment of developmental score and size at the 'hatch' stage (i.e. at 43 d.p.f at 10°C and at 149 d.p.f. at 2°C) suggested the existence of an effect of temperature on the gains made by the embryo from utilisation of the yolk (i.e. differences in yolk utilisation 'efficiency'). The timing of the 'hatch' stage was intended to be matched between embryos reared at 10°C and 2°C according to their level of development. However, mean developmental scores, as calculated based on examination of fixed specimens, were slightly higher in fish sampled at the 'hatch' stage at 10°C than at 2°C ($P<0.001$), by approximately 10 points (equivalent to 1% of the developmental sequence from fertilisation to first feeding) (Fig. 3.1a). There was no significant difference in mean body mass between 'hatch' samples at the two temperatures (Fig. 3.1b). However, although embryos reared at 10°C were more advanced and had similar body mass to those at 2°C, they had utilised significantly less of their endogenous yolk supply (Fig. 3.1c). Mean standard lengths at 'hatch' were lower at 10°C than at 2°C ($P<0.001$) (results not shown).

The gross quantity of yolk utilised at the 'hatch' stage also varied significantly with initial egg size ($P<0.001$). Embryos from relatively larger eggs had used more yolk, and had achieved higher mean values of developmental score, standard length and body mass, than those from smaller eggs ($P<0.001$ for all) (Fig. 3.1a-c). Significant interactions between embryonic temperature and egg size were found for developmental score ($P=0.026$), standard length ($P=0.004$) and quantity of yolk used ($P<0.01$). The magnitude of the effect of egg size on developmental score was greater at 10°C (Fig. 3.1a), while the impact of egg size on standard length and on the quantity of yolk used was greater at 2°C (Fig. 3.1c).

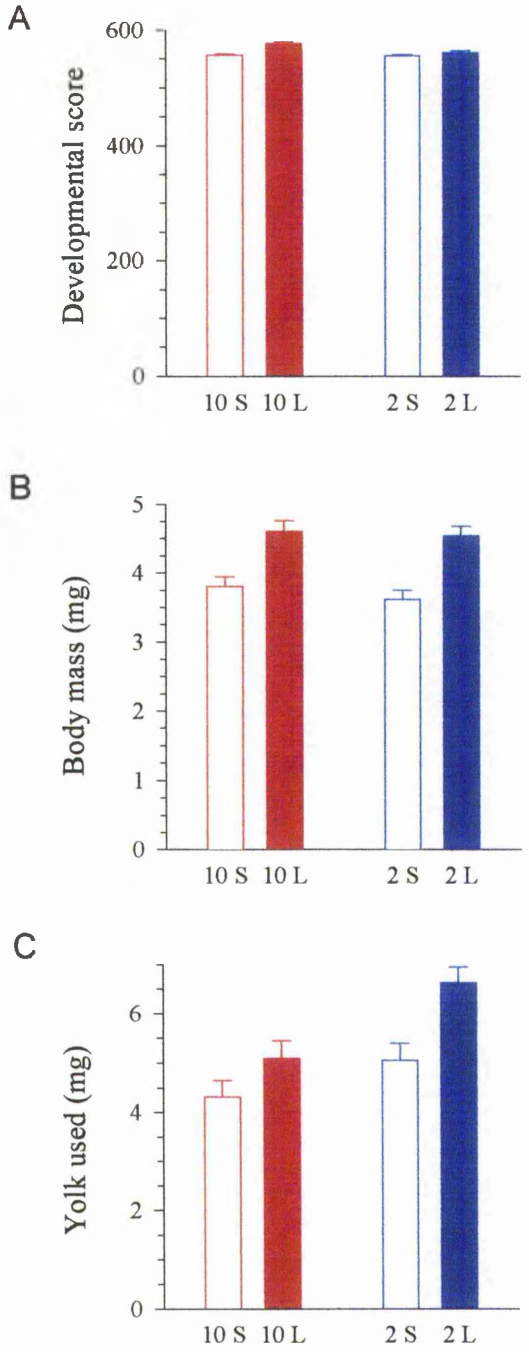



**Fig. 3.1.** Mean values of (**A**) developmental score, (**B**) body mass and (**C**) quantity of yolk utilised at the 'hatch' stage (43 d.p.f. at 10°C, 149 d.p.f. at 2°C). In order to illustrate the variation in these values with egg size, mean values are presented for embryos from the maternal groups of smallest egg size ('S', initial dry mass of yolk = 24.3mg) and of largest egg size ('L', initial dry mass of yolk = 36.8mg) at each temperature. '10' = 10°C, '2' = 2°C. n = 8 embryos for each maternal group at each temperature. Error bars are standard errors.

***Effects of temperature on patterns of yolk utilisation during the alevin period***

After the 'hatch' stage, the relationship between developmental score and the quantity of yolk used was significantly affected by both embryonic and post-'hatch' temperature (Table 3.1). The predicted relationship between score and yolk used at each of the four temperature regimes, for an alevin from an egg of dry mass 30mg (the mean egg size for all six maternal groups studied), is illustrated in Fig. 3.2a. (Note: Although the data in Figs. 3.2 and 3.3 may appear highly scattered, this is to a large extent due to the effects of egg size on efficiency (see below)). There was a clear trend for an increase in the developmental score achieved from a given quantity of yolk with increasing temperature.

The length achieved by an alevin from a given quantity of yolk increased significantly with post-'hatch' temperature (Fig. 3.2b, Table 3.1). The greatest difference was between the groups held at constant 10°C and constant 2°C. The temperature experienced by the fish during the embryonic period had no significant effects on the relationship between standard length and the quantity of yolk used.

The 'efficiency' with which the yolk supply was used to produce increases in body mass varied significantly with both embryonic and post-'hatch' temperature (Fig. 3.3a, Table 3.1). The optimum thermal regime for conversion of yolk into body mass was that experienced by the '2→6°C' group, while the fish in the constant 10°C group showed the lowest efficiency (Table 3.2). In addition, embryonic temperature interacted significantly with post-'hatch' temperature ($P<0.001$) to affect the conversion of yolk into body mass. When transferred to a common temperature (6°C) after the 'hatch' stage, alevins which had been reared at 2°C as embryos used their remaining yolk to increase body mass more efficiently than did alevins which had been reared at 10°C as embryos.

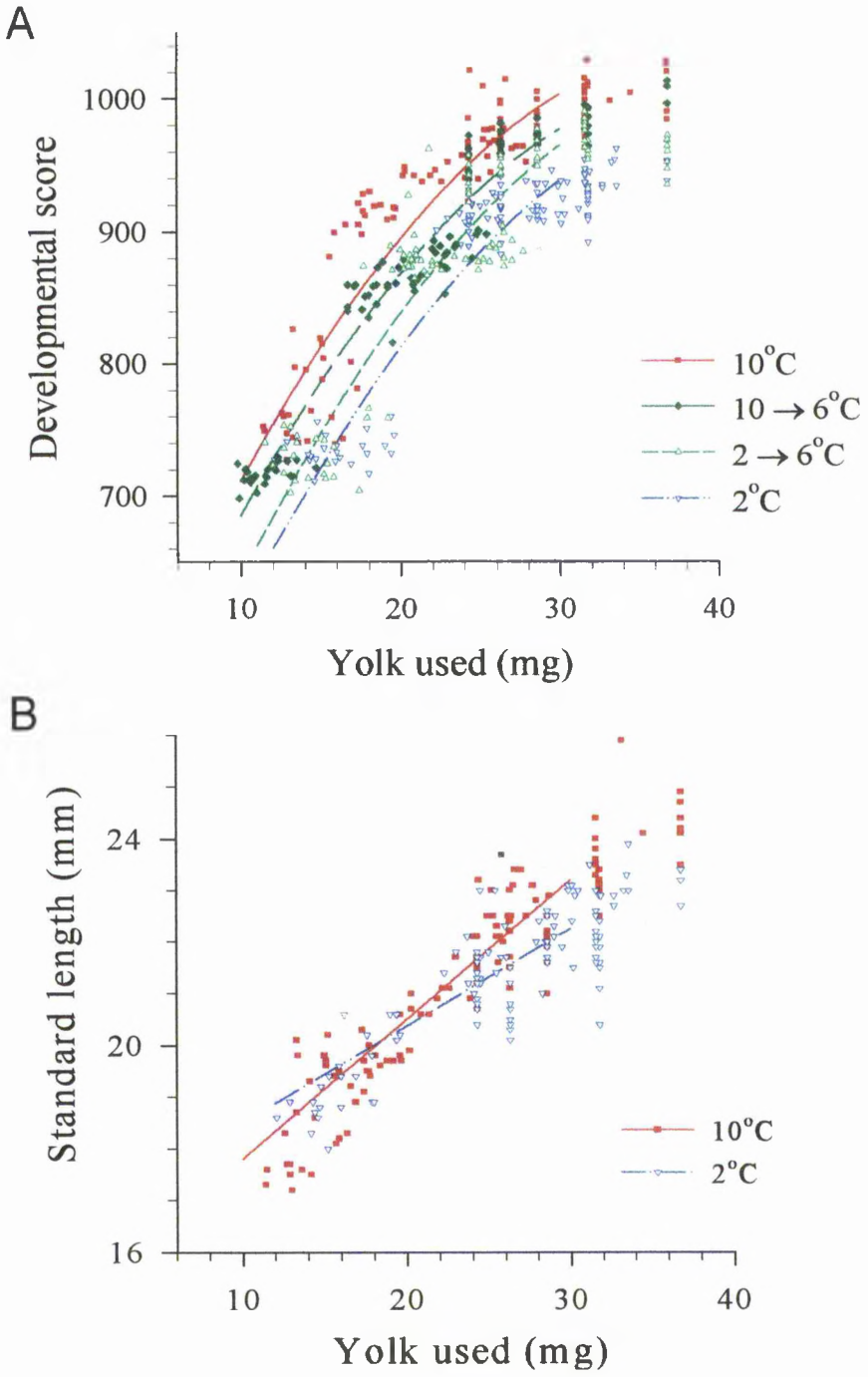



**Fig. 3.2.** Plots of **(A)** developmental score and **(B)** standard length, relative to the quantity of yolk used. The lines depict predicted values for an alevin from a 30mg egg reared under different temperature regimes, based on the regression equations in Table 3.1. For the sake of clarity, the plot of standard length against yolk used shows values at constant 10°C and constant 2°C only.

**Fig. 3.3.** Plots of (**A**) dry body mass relative to the quantity of yolk used, (**B**) standard length against developmental score, and (**C**) dry body mass against developmental score. The lines depict predicted values for an alevin from a 30mg egg reared under different temperature regimes, based on the regression equations in Table 3.1. For the sake of clarity, the plot of body mass against yolk used shows values only for alevins reared at constant 10°C and for alevins reared at 2°C until 149 d.p.f. and then transferred to 6°C. The dashed line in **A** represents the body mass that would be achieved by 100% efficiency of conversion of yolk into tissue. In **C**, the uppermost of the three lines represents the regressions for the 2°C and 2→6°C groups, which run coincidentally.

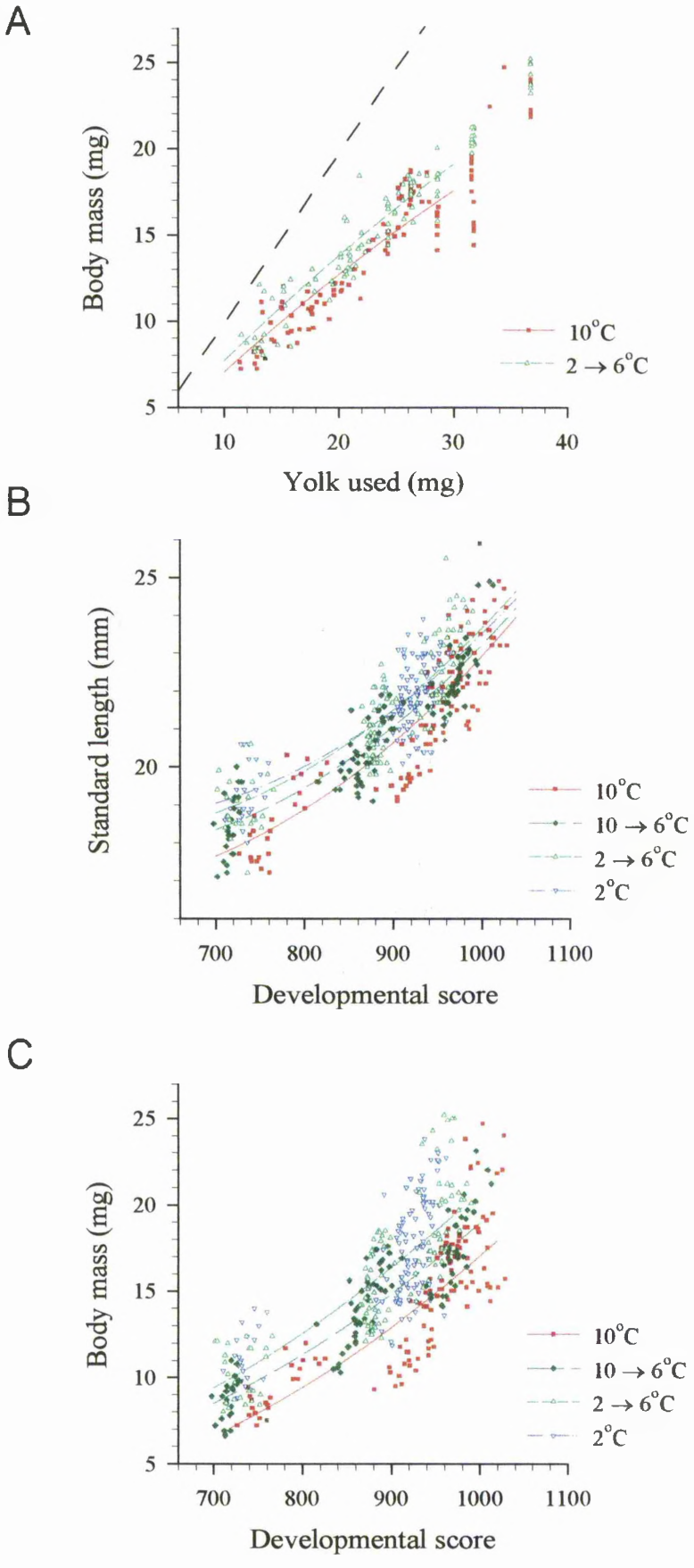

A
Body mass (mg)
25
20
15
10
5
10
20
30
40
Yolk used (mg)
10°C
2 → 6°C
B
Standard length (mm)
25
20
700
800
900
1000
1100
Developmental score
10°C
10 → 6°C
2 → 6°C
2°C
C
Body mass (mg)
25
20
15
10
5
700
800
900
1000
1100
Developmental score
10°C
10 → 6°C
2 → 6°C
2°C

**Table 3.1.** Regression equations for the relationships between the quantity of yolk utilised, developmental score, standard length, dry body mass and wet body mass.

| Y | X | Equation | $r^2_{adj}$ | Individual *P*-values | D.F. |
|---|---|---|---|---|---|
| Score (*S*) | Yolk used (*Y*) | $S = 523 + 31.3(Y) - 0.373(Y^2) - 31.0(T) - 6.29(E) + 3.22(T\text{x}E) - 1.77(Y\text{x}T) + 26.3(AT)$ | 92.2 | 0.04 (*T*), <0.001 for others | 7, 429 |
| Length (*L*) | Yolk used (*Y*) | $L = 11.9 + 0.334(Y) - 0.756(AT) + 0.157(E) + 0.0410(Y\text{x}AT) - 0.00487(Y\text{x}E)$ | 87.4 | <0.001 for all | 5, 430 |
| Dry body mass (*M*) | Yolk used (*Y*) | $M = 0.513(Y) - 0.00416(Y^2) + 0.0778(T\text{x}E) + 0.0440(Y\text{x}T) - 0.00749(Y\text{x}E) + 1.91(AT) - 0.0398(AT\text{x}E) - 1.63(AT\text{x}T)$ | 92.2 | 0.008 (*Y*x*T*), 0.001 (*AT*), 0.024(*AT*x*E*), <0.001 for others | 8, 422 |
| Length (*L*) | Score (*S*) | $L = 26.8 - 0.0333(S) + 0.000026(S^2) + 0.000134(S\text{x}E) - 1.98(AT) + 0.0253(AT\text{x}E) + 0.00133(S\text{x}AT) - 0.442(AT\text{x}T)$ | 89.5 | 0.006 (*AT*x*E*), 0.007(*S*x*AT*), <0.001 for others | 7, 440 |
| Dry body mass (*M*) | Score (*S*) | $M = 19.5 - 0.0487(S) + 0.000034(S^2) + 7.60(T) - 0.478(E) - 0.147(T\text{x}E) - 0.00313(S\text{x}T) + 0.00108(S\text{x}E) - 1.92(AT\text{x}T)$ | 90.1 | 0.012 (Constant), 0.003 (*S*), 0.04(*S*x*T*), <0.001 for others | 8, 432 |
| Dry body mass (*M*) | Length (*L*) | $M = -36.2 + 4.81(L) - 0.128(L^2) - 1.55(E) + 0.0853(L\text{x}E) + 3.39(AT) - 0.121(AT\text{x}E) - 0.611(AT\text{x}T)$ | 88.3 | <0.001 for all | 7, 432 |
| Wet body mass (*WM*) | Dry body mass (*M*) | $WM = 41.5 + 7.38(M) - 0.262(M^2) - 2.74(E) - 0.508(M\text{x}T) + 0.214(M\text{x}E) - 6.73(AT) - 0.340(AT\text{x}E)$ | 95.5 | 0.017(*AT*), <0.001 for others | 7, 432 |

D.F. = Degrees of freedom associated with the regression and with the error. E = Egg size. Length = Standard length. Score = Developmental score. All regressions had significance values of P<0.001.

**Table 3.2.** Predicted values for the efficiency of conversion of yolk into body mass at the point of complete exhaustion of the yolk.

| Temperature | Embryos | 10°C | | 2°C | |
|---|---|---|---|---|---|
| experienced as: | Alevins | 10°C | 6°C | 6°C | 2°C |
| | Egg Size | | | | |
| Conversion | Small[a] | 57.1 | 59.9 | 63.3 | 59.4 |
| efficiency (%) | Large[b] | 60.5 | 63.7 | 64.8 | 63.6 |

[a] Initial dry mass of yolk = 24.3mg. [b] Initial dry mass of yolk = 36.8mg. (These are the mean egg sizes for the maternal groups with the smallest and largest eggs respectively.)

Overall, the efficiency with which yolk was converted into tissue decreased during development. Combining temperature regimes and maternal groups, approximately 79% of the yolk used by the 'hatch' stage had been converted into body mass, whereas the mean efficiency for the combined final samples taken at each temperature was just 61%.

The differing effects of temperature on the use of yolk to produce increases in developmental score, length and body mass resulted in varying relationships between these parameters with temperature regime. Fish reared in the colder temperature regimes - constant 2°C and '2→6°C' - were longer relative to developmental score (Fig. 3.3b), and heavier relative to score (Fig. 3.3c), than fish at the '10→6°C' or constant 10°C regimes (Table 3.1). The mass of the fish relative to its standard length also increased with decreasing temperature. According to the regression model, an alevin of length 22mm, from a 30mg egg, would have a body mass of 15.8mg if reared at constant 10°C, but would weigh 17.5mg if reared under the '2→6°C' regime.

***Effects of egg size on patterns of yolk utilisation during the alevin period***

The gains achieved by an embryo from a given quantity of yolk varied with egg size (Table 3.1); the nature of this variation was different for advances in development and for increase in size. Alevins from smaller eggs were more advanced (Fig. 3.4a), but shorter (Fig. 3.4b), after use of a given quantity of yolk, than alevins from relatively larger eggs.

Alevins from larger eggs were also substantially more efficient at converting yolk into body mass than those from smaller eggs, at all temperatures studied; egg size appeared to be a more important determinant of conversion efficiency than was temperature (Fig. 3.4c). The interactions between egg size and temperature regime were complex in their effects on efficiency of yolk use for body mass, although the difference in efficiency with egg size was lowest in the '2→6°C' group.

Large egg size had similar effects to those of low temperature on the relationships between developmental score, length and body mass. Alevins from larger eggs were longer, and had greater body mass, at a given score, and were also heavier relative to length, when compared to alevins from small eggs. According to the regression model, the mean body mass of an alevin of length 22mm, reared under the '2→6°C' regime, would range from 16.1mg to 18.6mg depending on initial egg size (assuming a range in egg size of 24.3mg to 36.8mg, the range in mean egg sizes among the six female parents used in this study).

In all the relationships examined, use of the variable of egg size ($E$) consistently produced regressions with substantially greater values of goodness of fit than use of the variable of maternal group ($G$).

The findings described above are based on values of dry mass of yolk and of alevins. Examination of the relationship between alevin dry mass and wet mass revealed that the water content of the alevin increased with increasing egg size and decreasing temperature. Thus, comparing alevins which had attained a dry body mass of 16mg, the predicted wet body mass varied from 104mg for an alevin from a small

egg (egg size = 24.3mg) reared at constant 10°C, to 118mg for an alevin from a large egg (egg size = 36.8mg) reared under the '2→6°C' regime.

***Development and growth at the point of complete exhaustion of the yolk***

Examination of alevins in the final samples taken at each temperature revealed no significant relationship between initial egg size and the quantity of yolk remaining. In other words, although alevins from relatively larger eggs began life with a greater gross quantity of yolk than those from smaller eggs, they utilised these endogenous reserves at a greater rate, so that exhaustion of the yolk should occur at a similar time in all maternal groups. Although many of the alevins in the final samples had reached a point at which the remaining yolk was too small to be effectively quantified, others still had as much as 7.4mg of yolk remaining. For this reason, the developmental score, length and body mass of the alevin at the point of complete exhaustion of the endogenous reserves, as presented in Fig. 3.5, have been estimated based on the regression models, rather than measured directly from the final samples.

For all three parameters studied - developmental score, length and body mass - the greater gross quantity of yolk supplied to alevins from large eggs enabled them to achieve higher values; at the point of yolk exhaustion, alevins from large eggs were more advanced, longer and heavier (Fig. 3.5a-c). However, the effects of egg size on final developmental score were substantially smaller than those on the two growth parameters studied. This is at least partly due to the fact that large egg size is associated with lower rates of differentiation per unit yolk used, but with higher rates of growth per unit yolk used.

While the effects of temperature and of egg size on final developmental score were broadly comparable in magnitude, it can be seen from Fig. 3.5 that the length and mass achieved by the alevin were much more strongly related to egg size than to temperature.

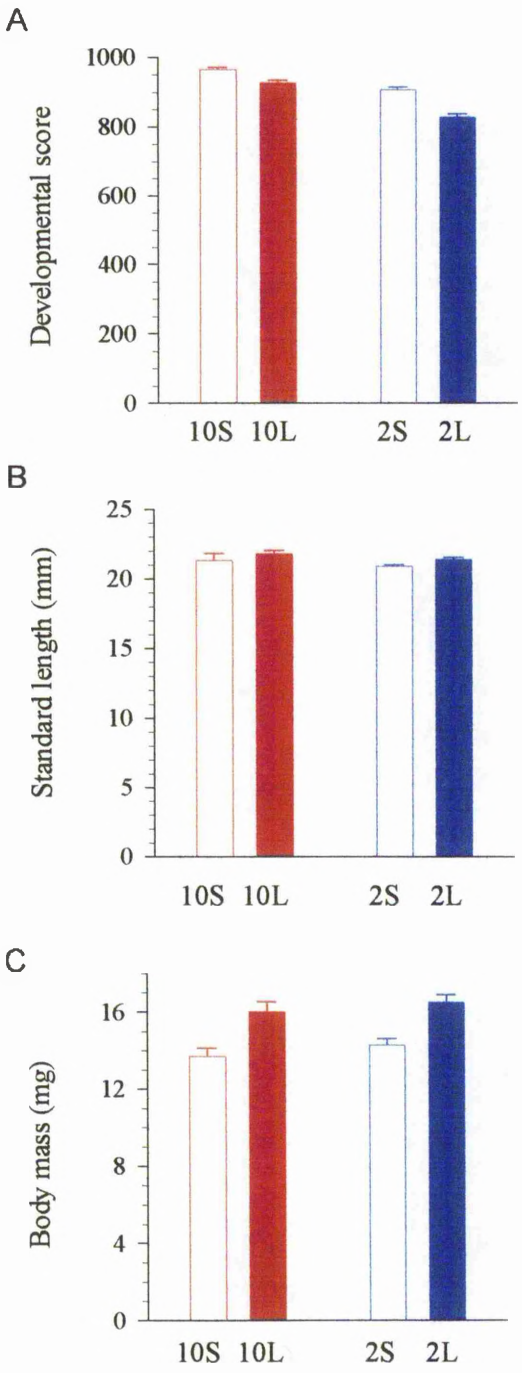
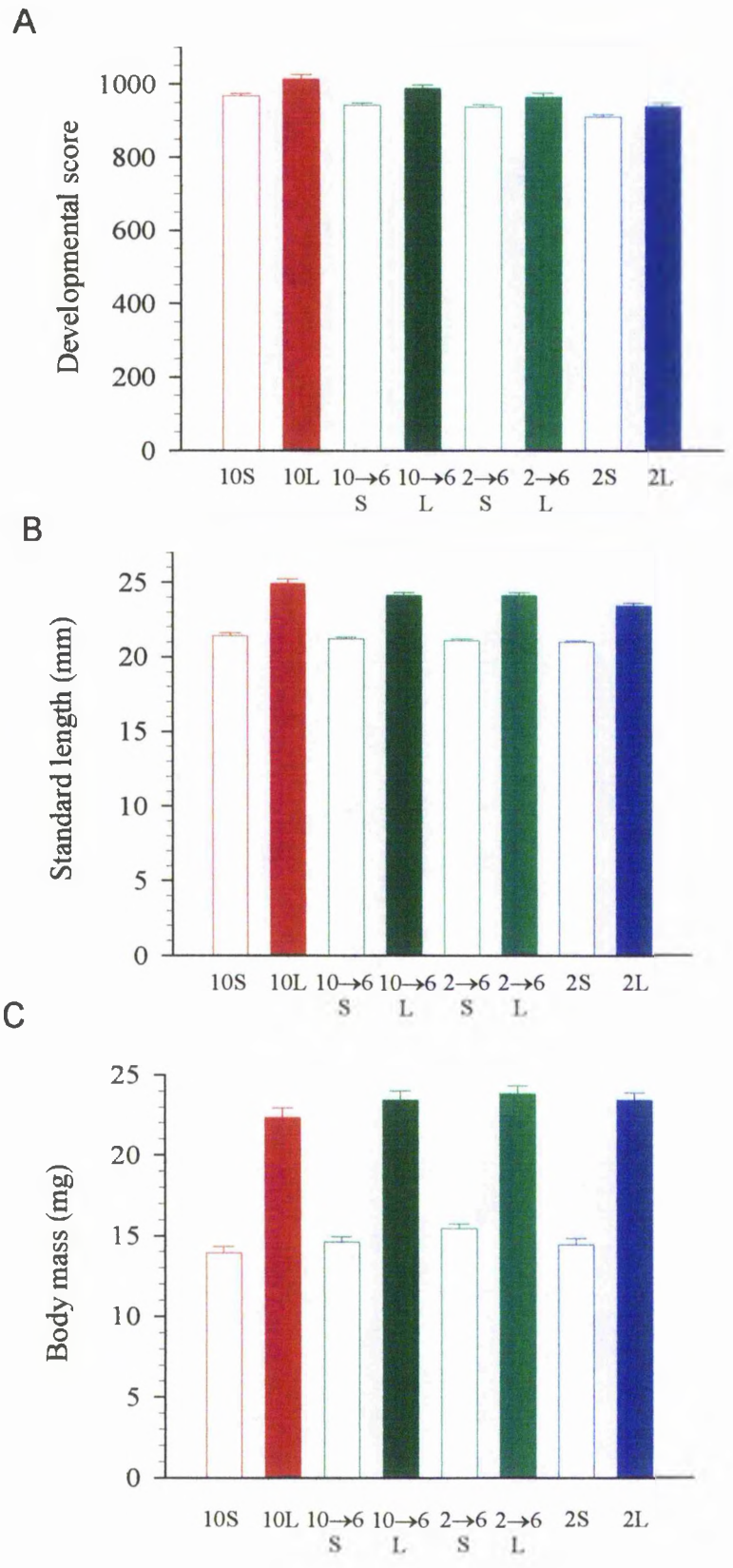



**Fig. 3.4.** Predicted mean values of **(A)** developmental score, **(B)** standard length and **(C)** dry body mass, after utilisation of 24mg of yolk. In order to illustrate the variation in these values with egg size, mean values are presented for embryos from the maternal groups of smallest egg size ('S', initial dry mass of yolk = 24.3mg) and of largest egg size ('L', initial dry mass of yolk = 36.8mg) at each temperature. Values are calculated according to the regression equations in Table 3.1. Error bars are 95% confidence intervals.

**Fig. 3.5.** Predicted mean values of (**A**) developmental score, (**B**) standard length and (**C**) dry body mass, at the point of complete exhaustion of the yolk, at four different temperature regimes: constant 10°C ('10'), 10°C until 43 d.p.f. and then 6°C ('10→6'), 2°C until 149 d.p.f. and then 6°C ('10→6'), and constant 2°C ('2'). In order to illustrate the variation in these values with egg size, mean values are presented for embryos from the maternal groups of smallest egg size ('S', initial dry mass of yolk = 24.3mg) and of largest egg size ('L', initial dry mass of yolk = 36.8mg) for each temperature regime. Values are calculated according to the regression equations in Table 3.1. Error bars are 95% confidence intervals.


A
Developmental score
1000
800
600
400
200
0
10S
10L
10→6 S
10→6 L
2→6 S
2→6 L
2S
2L
B
Standard length (mm)
25
20
15
10
5
0
10S
10L
10→6 S
10→6 L
2→6 S
2→6 L
2S
2L
C
Body mass (mg)
25
20
15
10
5
0
10S
10L
10→6 S
10→6 L
2→6 S
2→6 L
2S
2L

## Discussion

The efficiency with which yolk was utilised for growth by trout alevins varied significantly with both temperature and egg size. Conversion of yolk into body mass displayed a general trend of increased overall efficiency at lower temperatures (Fig. 3.5, Fig. 3.6, Table 3.2), in agreement with most previous studies on yolk utilisation in salmonids (Hamor & Garside 1977; Heming 1982; Murray & Beacham 1986; Murray & McPhail 1987; Rombough 1987). It has been suggested that this phenomenon is the result of expenditure of a relatively greater proportion of energy reserves on metabolic maintenance and physical activity at high temperatures (Hamor & Garside 1979); Fukuhara (1990) reported that the maximum level of physical activity observed in embryos of three marine species increased with an increase in rearing temperature. It has not yet been proven, however, that levels of activity increase disproportionately with temperature, relative to increases in the rates of growth and differentiation. Studies comparing patterns of yolk utilisation in free and artificially immobilised embryos (e.g. by use of toxins specific to motor neurons, or cutting the spinal cord) may provide some insight into this possibility.

Interestingly, the effects of temperature on efficiency of yolk use for increases in body mass appeared to differ between embryonic and alevin periods, with efficiency prior to the 'hatch' stage being higher at 10°C than at 2°C. Beacham & Murray (1985) reported similar findings for chum salmon (*Oncorhynchus keta*). However, fish reared at 10°C as embryos subsequently exhibited reduced overall efficiency compared to those reared at 2°C, even when both groups were transferred to a common temperature at the 'hatch' stage. Yolk contains a variety of components necessary for growth, such as neutral lipids and protein, and teleost embryos may utilise these components in different proportions depending on the stage of development (Ronnestad *et al.* 1998). If differing environmental temperatures also result in differences in the proportion of each component utilised at a given stage, then patterns of yolk usage during the embryonic period may determine the composition of the remaining yolk, and thus modulate efficiency of yolk utilisation during the alevin period.

Overall, utilisation of endogenous reserves to increase body mass was most efficient in fish reared at 2°C as embryos and then transferred to 6°C. It is known that the temperature regime resulting in optimum yolk usage in a given species usually closely matches that experienced by that species in the wild (Murray & McPhail 1987; Hendry *et al.* 1998). Trout embryos normally experience low temperatures during the winter, prior to hatching, and then experience a gradual increase in ambient temperature during the alevin period; the '2→6°C' regime is thus likely to most closely mimic the natural thermal regime experienced by trout spawned in the Don river. However, while rearing under the '2→6°C' regime maximised the body mass of the alevin at yolk exhaustion, efficiency of yolk use for increase in length was highest in alevins reared at constant 10°C. Differences in the response to temperature of increases in length and in mass have also been found in other salmonid species (Beacham & Murray 1985; Murray & Beacham 1986), suggesting a degree of independence of regulation of the two processes. This is supported by the finding that the temperature experienced prior to the 'hatch' stage affected efficiency of yolk use for increases in mass but not in length.

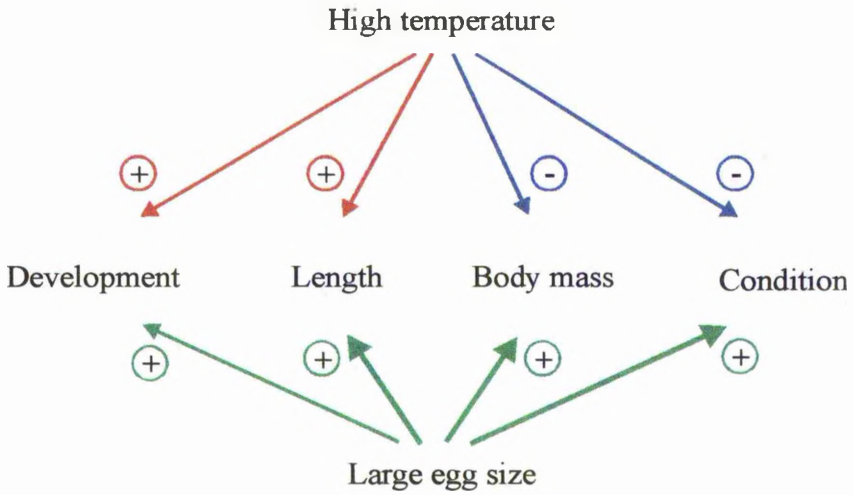



**Fig. 3.6.** Schematic illustrating the effects of relatively high temperature and of relatively large egg size on the degree of development, standard length, body mass and condition (i.e. mass relative to length) of alevins at the point of complete exhaustion of the yolk. ⊕ Positive effect. ⊖ Negative effect. The effects of temperature as depicted refer to general trends for the entire period from fertilisation to yolk exhaustion.

While previous studies on yolk utilisation have concentrated almost exclusively on the growth of the embryo and alevin, the present study has also examined the development of the fish, that is, the process of differentiation, as opposed to increase in size. Use of the scoring system described in Chapter 2 meant that the degree of differentiation of specimens was judged based on simultaneous examination of as many as 14 separate morphological features, providing much greater precision than in previous studies. The pattern of utilisation of the yolk supply to produce advances in development was found to vary with both environmental temperature and egg size, and in ways quite distinct from the responses observed in the growth parameters studied. Maximum conversion of yolk into increases in development was observed in small eggs (rather than in large eggs, as was the case for both length and body mass) reared at relatively high temperatures (unlike the lower temperatures which generally gave improved conversion efficiencies of yolk into mass). Attainment of certain developmental features, such as a functional jaw, has also been found to occur with less gross expenditure of yolk at higher temperatures in other species, such as the Japanese flounder (*Paralichthys olivaceus*) (Fukuhara 1990), the wolffish (*Anarhichas lupus*) (Pavlov & Moksness 1995) and the sole (*Solea solea* L.) (Baynes & Howell 1996). In addition, it is known that the quantity of yolk remaining to alevins when they emerge from the gravel redd is greater in alevins of Atlantic salmon (*Salmo salar*) and chinook salmon (*Oncorhynchus tshawytscha*) reared at high temperatures than in those reared at low temperatures (Heming 1982; Brännäs 1988). If the same holds true of *S. trutta*, then the timing of emergence may well be determined by attainment of a critical level of development, rather than a certain minimum body mass.

For all parameters studied, the effects of the differences in efficiency of yolk usage with temperature were equalled or outweighed by the effects of differences in the quantity of yolk between alevins from large and small eggs. Alevins from relatively larger eggs utilised a greater quantity of yolk per unit time than those from small eggs, and achieved a greater level of developmental advancement at a given age. Although a similar increase in developmental rate with increasing egg size has been reported for Icelandic cod (*Gadus morhua*) (Marteinsdottir & Able 1992), most studies have found that egg size has no effect on the rate of differentiation (Blaxter & Hempel 1963; Kazakov 1981; Thorpe *et al.* 1984; Beacham & Murray 1985; Baynes

& Howell 1996), or that embryos from large eggs develop more slowly (Kamler & Kato 1983; Chambers *et al.* 1989). However, the effects of egg size on the rate of development have most commonly been examined based on measurements of the time at which 50% of the embryos have hatched, which has in any case been shown to vary with environmental conditions (Peterson *et al.* 1977; Heming 1982; see also Chapter 2).

At the point of complete exhaustion of the yolk, the magnitude of variation in developmental score with temperature and with egg size was approximately equal (Fig. 3.5). In contrast, growth parameters such as standard length and body mass varied far more with egg size than with temperature. Previous studies have found larger egg size to be associated with increases in size at and after first feeding (Fowler 1972; Pitman 1979; Hutchings 1991; Ojanguren *et al.* 1996), as well as increases in the efficiency of food conversion (Pitman 1979), in swimming endurance (Ojanguren *et al.* 1996) and in rates of survival after emergence (Hutchings 1991). Rates of survival prior to yolk exhaustion have also been reported to vary with egg size, and with temperature, although the nature of these variations differs substantially between studies (e.g. Bagenal 1969; Fowler 1972; Beacham & Murray 1985; Brännäs 1988). The difference in size between fish arising from eggs of different sizes may persist for as long as twelve weeks after first feeding (Fowler 1972); differences in length of as little as 1mm may affect the position of young fish in territorial hierarchies (Chapman 1962). An increase in maternal investment in the form of greater mean egg size would therefore appear to substantially increase the fitness of individual alevins. However, for a female parent of a given size, there is a necessary trade-off between large egg size and high fecundity (Springate & Bromage 1984; Elgar 1990; Quinn *et al.* 1995). The effects of temperature on efficiency of yolk use, although relatively small, may affect the relative advantages of producing few, large offspring or many, small offspring.

# Chapter 4: Temperature and neuromuscular development in trout embryos

## Introduction

Recent studies have indicated that anadromy in the trout (*Salmo trutta*) may have a heritable basis, related to maternal phenotype (Clarke *et al.* 1994; Thompson 1995) and reflected in the inheritance of mitochondrial DNA variation (Thompson 1995). Environmental temperature may also play a role, possibly by interacting with genotype to affect patterns of embryonic development, although the experimental evidence in support of this idea is somewhat contradictory. Gorodilov (1989) found no effects of temperature or parental migratory type on the relative timings of early ontogenetic changes in *S. trutta*. However, Halacka (1995) reported that certain developmental events, such as otic vesicle formation, began at later somite stages in offspring of freshwater resident trout reared at a mean temperature of 1.6°C, than in offspring of sea trout reared at 4.8°C as reported by Pavlov (1989).

Salmonid embryos begin moving spontaneously within the egg envelope well before hatching. Such movements are believed to improve perivitelline circulation, and thus gas exchange (Peterson & Martin-Robichaud 1983), and may contribute to coordination of subsequent neural development (Lømo & Slater 1980; Rubin *et al.* 1980). The onset of such muscular activity is contingent on the differentiation of myoblasts into myotubes, which produce contractile myofibrils. Myogenesis is believed to be regulated by diffusible factors released from both the spinal cord and notochord (Buffinger & Stockdale 1994; Pownall *et al.* 1996; Blagden *et al.* 1997). Even when the muscle fibres contain large numbers of myofibrils, neural input may also be required to stimulate muscle contraction. Primary motor neurons produce axons which emerge from the spinal cord and follow specific pathways along the myosepta between adjacent somites (Eisen *et al.* 1986). Pathfinding by these axons is at least partly mediated by expression of neural cell adhesion molecules (NCAM) on the surface of the axons and on the myosepta themselves (Metcalfe *et al.* 1990; Somasekhar & Nordlander 1995). At the sites of contact between axons and trunk

muscle fibres, functional endplates develop, indicated by the accumulation of acetylcholinesterase; this molecule breaks down the neurotransmitter acetylcholine (Potter 1970), preventing desensitisation of the synapses (Kullberg *et al.* 1990).

Studies on species such as the Atlantic herring (*Clupea harengus*) have shown that environmental temperature can affect many aspects of embryonic development, from the relative timings of organ formation to the dynamics of muscle growth (Johnston *et al.* 1995). Many of the steps in neuromuscular development described above are also variable with temperature. Myofibril production, primary motor neuron axonogenesis, and acetylcholinesterase production all begin later, relative to somite stage, in herring embryos reared at 5°C when compared to those reared at 12°C (Hill & Johnston 1997b; Johnston *et al.* 1997), and temperature-induced differences in the patterns of muscle innervation can persist into the larval stages (Johnston *et al.* 1997). As reported in Chapter 2, trout embryos reared at low temperatures begin spontaneous muscle contractions later with respect to somite stage. The aim of the study described in this chapter was therefore to examine patterns of muscular and neural development in trout embryos, and to describe the effects of environmental temperature and of maternal migratory type.

## Materials and Methods

The trout (*S. trutta*) embryos examined were produced from the October 1995 fertilisation described in Chapter 2 (see p. 51-52; also see Appendix I). Each maternal group from this fertilisation was subdivided among egg trays supplied with river water at three controlled temperatures: 10°C (9.8-10.4°C), 6°C (5.6 - 6.2°C) and 2°C (1.6-2.4°C). The migratory type of each female parent was identified on the basis of the carotenoid pigment content of unfertilised eggs (5 - 10 eggs per female), after Noack *et al.* (1996) (See Appendix II).

Embryos were sampled daily at 10°C, once every two days at 6°C, and once every four days at 2°C, from early somitogenesis (Step S2) to the time of appearance of body pigmentation (step Y4). The eggs were placed in phosphate-buffered saline (PBS); once embryos had progressed beyond the 40-somite stage, MS-222 (ethyl m-

aminobenzoate) was added to a concentration of approximately 0.02% (w/v). The embryos were dissected out of the egg capsules using sharpened No. 5 watchmakers' forceps and the yolk sacs were removed.

Specimens to be used for study of myogenesis were fixed overnight in Bouin's fixative, then stored in 70% ethanol; sagittal wax sections were cut at a thickness of 8μm and stained with Mayer's haemalum and eosin. The positions of the somites containing the most posterior myotubes, and the most posterior muscle fibres with striations visible with a light microscope, were noted.

Specimens used for examination of neural development were fixed overnight in buffered 10% formalin (pH 7.2), then transferred to 0.1% sodium azide in PBS. For immunohistology, embryos were washed in PBS (2 x 5 mins), stored at 4°C overnight in 1% Triton X-100 (Sigma), and washed again in PBS (2 x 5 mins). The embryos were then alternately washed in distilled water at room temperature and in 100% acetone at -20°C (7 mins each), three to four times; this step increased permeability of the embryos. Non-specific binding sites were blocked by incubation in 10% normal goat serum (Sigma) / 1% bovine serum albumen (BSA) (Sigma) / 1% Triton X-100 in PBS (20 mins). The primary antibodies used were mouse monoclonal antibody HNK-1 (Sigma), which recognises a carbohydrate moiety in the N-CAM and L2 cell adhesion molecules; and anti-α-acetylated tubulin mouse monoclonal antibody (Sigma), which labels all neural processes. After overnight incubation in PBT (1% BSA / 1% Triton X-100 in PBS) containing primary antibody diluted 1:1000, the specimens were washed six times in PBT over a two-hour period, followed by incubation in secondary antibody. The secondary antibodies used were: HRP-conjugated goat anti-mouse IgM, for HNK-1 (diluted to 1:1000 in PBT, overnight incubation); FITC-conjugated goat anti-mouse IgG, for anti-α-acetylated tubulin (diluted to 1:200 in PBT, four hours incubation). Unbound secondary antibody was removed by frequent washes in PBT (6 x 10 mins). Embryos labelled with HNK-1 were presoaked in 0.1M diaminobenzidine (DAB) in PBS for 15 mins; peroxidase was then detected by addition of $H_2O_2$ diluted to 0.15%. The staining reaction was stopped by washes in PBS (3 x 1 min). Specimens were mounted in Immumount (Shandon) under glass coverslips supported by a square of silicon

grease, and examined using a Leitz DMRB system microscope fitted with Nomarski differential interference contrast (DIC) optics. Embryos labelled for acetylated tubulin were examined under epifluorescence. The positions were noted of the most posterior HNK-1-positive myosept, the most posterior primary motor neuron axon (tubulin-labelled specimens) and the primordium of the posterior lateral line (both HNK-1- and tubulin-labelled specimens). Emergence of the dendrites of Rohon-Beard neurons (early-developing trunk sensory neurons) was also studied.

Examination of the development of acetylcholinesterase activity was carried out on formalin-fixed specimens. The embryos were washed in PBS (2 x 5 mins), incubated in 1% saponin in PBS (2 hours), and washed again (PBS, 3 x 5 mins). This was followed by incubation for 3 - 6 hours in the dark in a solution of: 1.7 $mmol.l^{-1}$ acetylthiocholine iodide, 3 $mmol.l^{-1}$ copper sulphate, 100 $mmol.l^{-1}$ maleate buffer, 0.5 $mmol.l^{-1}$ potassium ferricyanide, 5 $mmol.l^{-1}$ trisodium citrate; pH 7.2 (Karnovsky & Roots 1964). The embryos were then washed in PBS (3 x 5 mins), mounted as described above, and viewed under DIC optics. The position was noted of the most posterior somite adjacent to which acetylcholinesterase activity was visible in the spinal cord, and of the most posterior somite with a stained posterior myosept. In all embryos examined for neural development, the posterior extent of vacuolisation in the notochord was also recorded.

Embryos with 60 or fewer somites were staged according to total somite number. Those with more than 60 somites were considered to have progressed beyond the point at which somites are produced at a regular rate. Such embryos were staged according to their age, expressed as the number of somite-intervals after the beginning of somitogenesis (S.I.) (Hill & Johnston 1997b). One somite-interval is the mean time taken for the formation of one somite pair, at the relevant temperature. (Note: The developmental scoring system described in Chapter 2 was not available at the time when the present study was carried out.)

Linear regressions produced for the data were compared by analysis of covariance (ANCOVA) using temperature and maternal migratory type as factors and somite stage as a covariate, using the Minitab statistical analysis package (Minitab Inc., USA). Where temperature was found to have a significant effect at the

5% level, the data for the two migratory types were combined, and the data were examined for significant differences in the slopes of the regressions, using the first part of the ANCOVA technique described by Zar (1996). The second part of the ANCOVA was used to test for significant differences in the elevations of the regressions, followed by Tukey multiple comparison tests for pairwise comparisons of elevations.

## Results

### *Muscle development*

Newly formed somites consisted of undifferentiated cells, most of which lay with their nuclei adjacent to the intersomitic boundaries (Fig. 4.1a.). Myogenesis began in the most rostral somite, when the myoblasts at the medial boundary of the somite, immediately adjacent to the notochord and at the level of the horizontal midline, began to elongate, eventually spanning the length of the somite. These cells formed the mononucleate pioneer myotubes (Fig. 4.1b). Around the same time, adjacent myoblasts lined up in rows of two to five cells and fused to form multinucleate myotubes, also running the length of the somite. Production of the first myotubes was calculated to begin at approximately the 9-somite stage (Table 4.1, Fig. 4.2a,b). Myogenesis proceeded posteriorly down the body, as well as spreading in a medial-to-lateral direction through the myotome. By approximately the 30-somite stage, production of contractile filaments, indicated by the presence of cross-striations in the cytoplasm, had begun in the most anterior pioneer myotubes as they continued their development into muscle fibres (Fig. 4.1d, 4.2c,d). As well as the main medial zone of initial myogenesis, additional smaller zones were often seen more superficially, near the dorsal and ventral edges of the somites (Fig. 4.1d). These superficial zones, which appeared to contain mostly multinucleate but also some mononucleate myotubes, were eventually subsumed by the expansion of the medial zone.

The rates of progression down the body of myotube formation and development of cross-striations, with respect to somite stage, were not significantly affected by either temperature or maternal migratory type at the 5% level. Myotubes were formed in the most posterior somite by approximately 70 S.I. (Fig. 4.2a,b); striated muscle fibres were present by 80 S.I. (Fig. 4.2c,d).

***Neural development***

All myosepta were weakly immunopositive for the HNK-1 antibody at all stages studied. However, by the 35 - 40 somite stage the anterior-most myosepta labelled with markedly greater intensity, particularly at their ventral, and to a lesser degree, dorsal extremities (Fig. 4.1c). All myosepta exhibited this increase in HNK-1 immunoreactivity by 80 - 90 S.I. (Fig. 4.3a,b).

Primary motor neurons were only weakly labelled by the HNK-1 antibody, but displayed strong immunoreactivity to the anti-α-acetylated tubulin antibody. Axons emerged from the spinal cord at the ventral motor root, located towards the anterior boundary of the adjacent somite (Fig.4.1e). The axons then extended ventrally and also caudally, so that by the time the growth cone reached the ventral edge of the somite it was almost level with the posterior somite boundary. The first axons emerged from the spinal cord at approximately the 30 somite stage; by approximately 90 S.I., primary motor neuron axons were visible medial to all somites (Fig. 4.3c,d).

The timings of development of HNK-1-positive myosepta and of primary motor neuron axons, relative to the log of the somite stage, were similar regardless of temperature and maternal migratory type (Fig. 4.3a-d).

**Fig. 4.1. A:** Sagittal section through the tailbud of a 59-somite embryo.

**B:** Sagittal section through somites 36 - 39 of a 69 S.I. embryo. The section is slightly angled such that the right hand side is more lateral, so that pioneer mononucleated myotubes (pm) are visible in somite 37, while multinucleated myotubes (mm) are visible in somite 38. A superficial zone (sz) of myogenesis is visible at the dorsal edges of the somites.

**C:** Whole-mount 56-somite embryo labelled with the HNK-1 antibody. ms = myosepta. rb = dendrites of Rohon-Beard neurons.

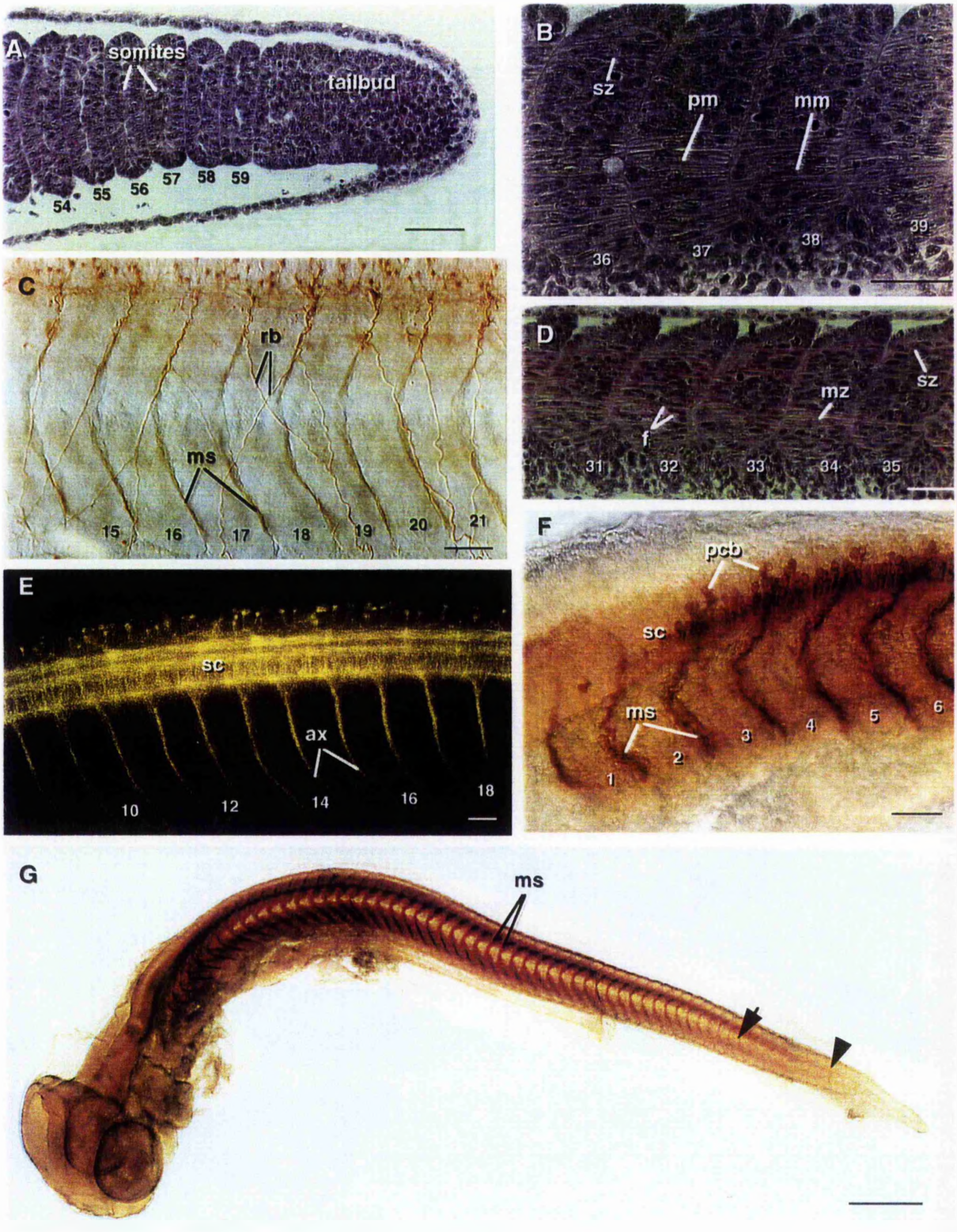
**D:** Sagittal section through somites 31 - 35 of a 76 S.I. embryo. Striations are visible in muscle fibres (f) in both the medial (mz) and superficial zones (sz) of myogenesis.

**E:** Whole-mount 84 S.I. embryo labelled with the anti-α-acetylated tubulin antibody, viewed under fluorescence. ax = axons of primary motor neurons. sc = spinal chord.

**F:** Whole-mount of a 58-somite embryo labelled for acetylcholinesterase. ms = myosepta. pcb = primary motor neuron cell bodies. sc = spinal chord.

**G:** 84 S.I. embryo labelled for acetylcholinesterase. The arrow indicates the posterior extent of labelling at the myosepta. The arrowhead indicates the posterior extent of labelling in the spinal chord. ms = myosepta.

In all photos, anterior is to the left, dorsal to the top. Numbers indicate somite number. Scale bar = 50μm (A - F), 0.25mm (G). Figs. D, F and G have been sharpened using Adobe Photoshop (Adobe Systems Inc., U.S.A.).


A
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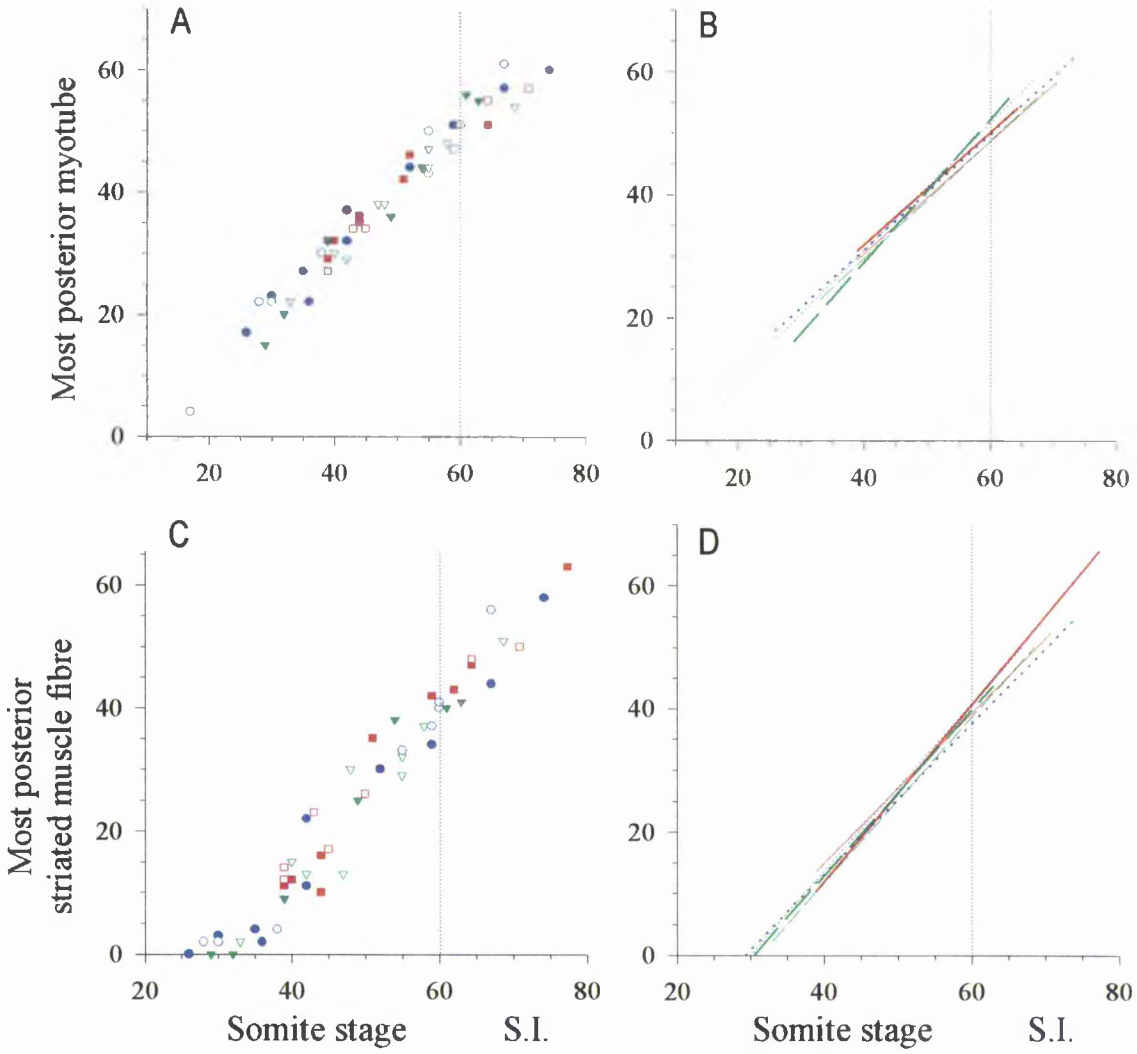
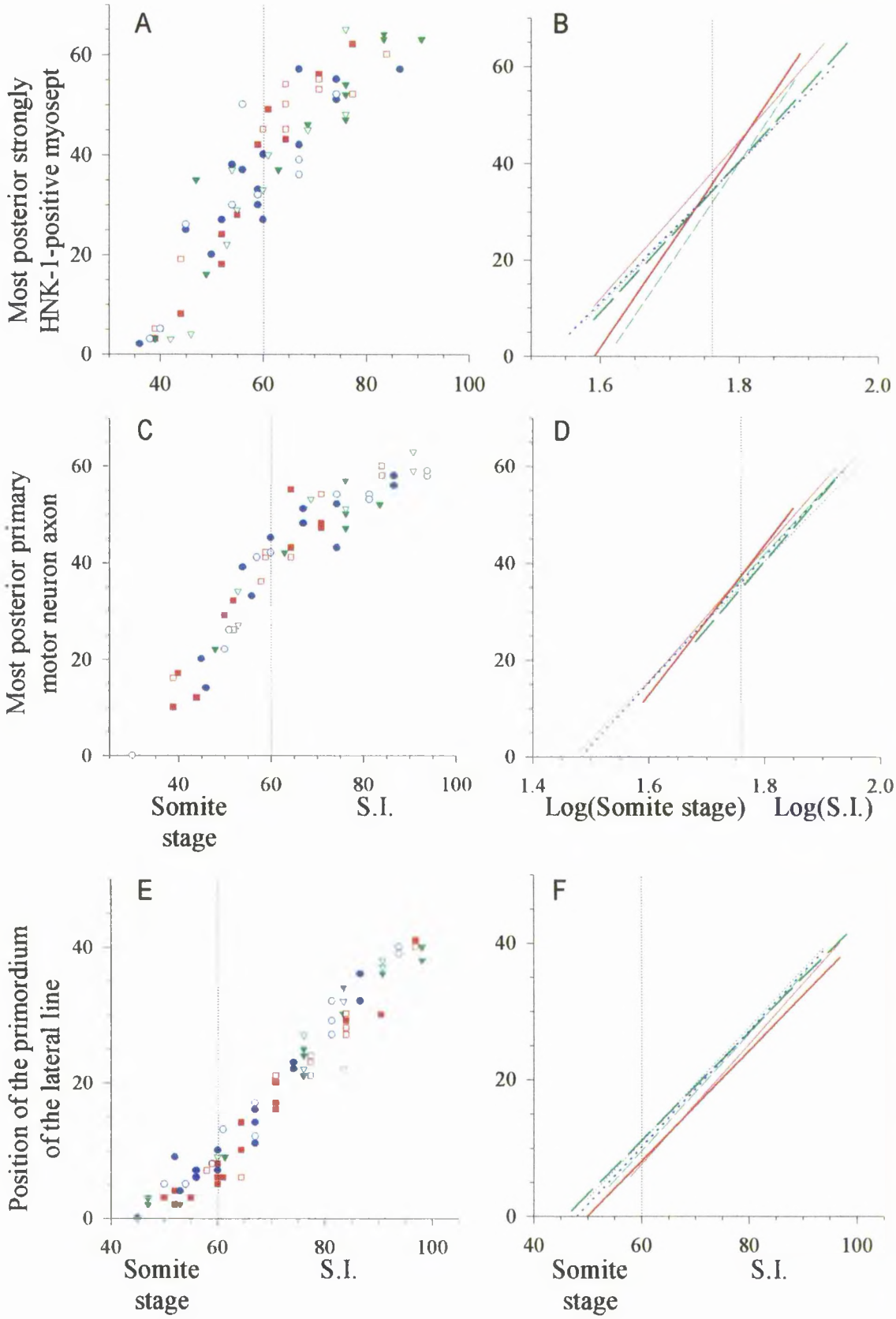



**Fig. 4.2.** Scattergrams and linear regression plots illustrating the anterior-posterior progression, in terms of number of somites, of formation of myotubes (**A** and **B**) and development of striations in the muscle fibres (**C** and **D**). S.I. = Embryo age, expressed as the number of somite-intervals after the beginning of somitogenesis. 10°C: Red, squares, solid lines. 6°C: Green, inverted triangles, dashed lines. 2°C: Blue, circles, dotted lines. Offspring of migratory females: solid symbols, bold lines. Offspring of non-migratory females: open symbols, regular lines.

**Fig. 4.3.** Scattergrams and linear regression plots illustrating the anterior-posterior progression, in terms of number of somites, of strong HNK-1 immunoreactivity at the myosepta (**A** and **B**), primary motor neuron axonogenesis (**C** and **D**) and the position of the lateral line primordium (**E** and **F**). Log (somite stage) / Log (S.I.) has been used as the x-axis for the linear regression plots in **B** and **D** due to the curved nature of the scattergrams in **A** and **C**. S.I. = Embryo age, expressed as the number of somite-intervals after the beginning of somitogenesis. 10°C: Red, squares, solid lines. 6°C: Green, inverted triangles, dashed lines. 2°C: Blue, circles, dotted lines. Offspring of migratory females: solid symbols, bold lines. Offspring of non-migratory females: open symbols, regular lines.


A
B
C
D
E
F
Most posterior strongly HNK-1-positive myosept
Most posterior primary motor neuron axon
Position of the primordium of the lateral line
Somite stage
S.I.
Log(Somite stage)
Log(S.I.)

The primordium of the posterior lateral line extended from a large ganglion posterior to the otic vesicle, and reached the level of the most anterior somite by the 45 - 50 somite stage. It progressed posteriorly at the rate of approximately 0.8 somites per S.I. (Table 4.1, Fig. 4.3e,f). Although labelled neural processes could be seen for most of the length of the newly-formed lateral line, they terminated just inside the anterior portion of the bulbous primordium. The primordium itself, approximately 2 - 3 somite widths long, was not immunopositive for either of the antibodies used. The labelled neural processes usually diverged into dorsal and ventral groups of neurites just proximal to the primordium. Although the posterior extension of the lateral line primordium was not affected by maternal migratory type, its progress relative to somite stage was affected by temperature ($P < 0.001$). At a given somite stage, the position of the primordium lagged approximately 2 - 3 somite widths behind in embryos reared at 10°C relative to those reared at 6°C ($P<0.01$) or at 2°C ($P<0.001$). The rate of posterior progression was not significantly affected by temperature.

Rohon-Beard neuron cell bodies and dendrites were labelled by both the HNK-1 and the tubulin antibodies. The large cell bodies were distributed continuously through the dorsal region of the spinal cord. Although some emergent dendrites passed over the tops of the adjacent myotomes to reach the epidermis, most were concentrated in fascicles in the clefts between the tops of the myotomes (Fig. 4.1c). Once the dendrites made contact with the epidermis, they extended ventrally, usually producing 2 - 4 branches which extended over the surfaces of several adjacent myotomes. Unlike primary motor neuron axons, which emerged in a strict anterior-posterior sequence, Rohon-Beard dendrites appeared in a highly imperfect order, often with gaps of five or even ten somite-widths between sequential dendrites. By the 50 somite stage, they were present to approximately the 35th somite, and were present to the most posterior somite by 80 - 100 S.I. (not shown). The timing of Rohon-Beard dendrite emergence was unaffected by either maternal migratory type or temperature.

***Endplate formation***

Embryos at the 50 somite stage exhibited acetylcholinesterase (AChE) activity in cell bodies in the spinal cord as far posteriorly as somite 30 (Fig. 4.4a,b). At this stage, AChE production also began between the more anterior somites, quite diffusely at first but becoming increasingly concentrated at the myosepta as the embryo developed (Fig. 4.1f,g). The number of cell bodies in the spinal cord producing AChE increased; the cell bodies of primary motor neurons could be distinguished from those of secondary motor neurons by their more dorsal location (Fig. 4.1f). Endplates containing AChE were present between all somites by 100 - 120 S.I. (Fig. 4.4c,d). The extent of AChE activity both in the spinal cord and at the myosepta, relative to the log of the somite stage, was not significantly affected by temperature or maternal migratory type.

***Notochord vacuolisation***

When the notochord was first formed, numerous vertical striations resulted in what is known as the appearance of a 'stack of pennies' (Kimmel *et al.* 1995). Midway through somitogenesis, cells within the notochord swelled to form large vacuoles. This process of notochord vacuolisation progressed in an anterior-posterior sequence, and was significantly affected by temperature ($P<0.001$) (Table 4.1, Fig. 4.4e,f). At a given somite stage, 10°C embryos had more advanced notochord vacuolisation than 6°C ($P<0.005$) embryos, which in turn had vacuoles to a greater posterior extent than 2°C embryos ($P<0.01$). The difference between the 10°C and 2°C groups amounted to a mean lag of approximately 4 - 8 somite widths at the lower temperature ($P<0.001$). The rate of progression of vacuolisation was not significantly different between temperature groups, and there was no significant effect of maternal migratory type.

**Fig. 4.4.** Scattergrams and linear regression plots illustrating the anterior-posterior progression, in terms of number of somites, of acetylcholinesterase (AChE) expression in the spinal chord (**A** and **B**) and in neuromuscular junctions at the myosepta (**C** and **D**), and of vacuolisation in the notochord (**E** and **F**). Log (somite stage) / Log (S.I.) has been used as the x-axis for the linear regression plots in **B** and **D** due to the curved nature of the scattergrams in **A** and **C**. S.I. = Embryo age, expressed as the number of somite-intervals after the beginning of somitogenesis. 10°C: Red, squares, solid lines. 6°C: Green, inverted triangles, dashed lines. 2°C: Blue, circles, dotted lines. Offspring of migratory females: solid symbols, bold lines. Offspring of non-migratory females: open symbols, regular lines.

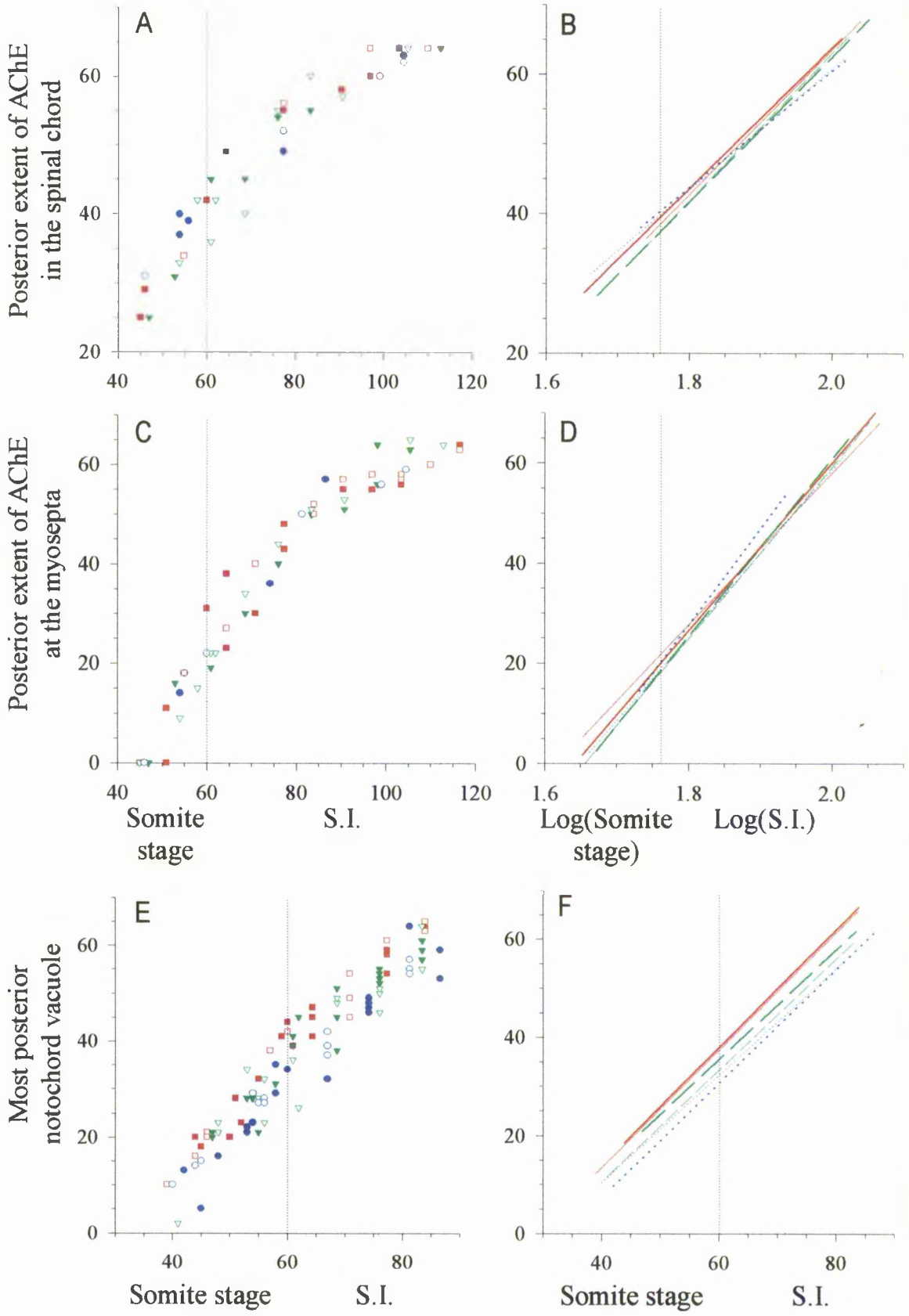

A
B
C
D
E
F
Posterior extent of AChE in the spinal chord
Posterior extent of AChE at the myosepta
Most posterior notochord vacuole
Somite stage
S.I.
Log(Somite stage)
Log(S.I.)
20
40
60
0
80
100
120
1.6
1.8
2.0

**Table 4.1.** Regression equations for the various aspects of myogenesis and neural development examined, relative to somite stage[a].

| Posterior extent of: | Relative to: | Slope | Intercept | $r^2$ (adjusted) | Residual D.F.[b] |
|---|---|---|---|---|---|
| Myotubes | Somite stage | 0.984 | -8.87 | 96.1 | 47 |
| Striated muscle fibres | Somite stage | 1.32 | -39.7 | 95.2 | 49 |
| HNK-1 immunoreactivity at myosepta | Log (Somite stage) | 169 | -263 | 86.8 | 65 |
| Axonogenesis of primary motor neurons | Log (Somite stage) | 132 | -195 | 92.6 | 53 |
| Lateral line primordium - | Somite stage | | | | |
| 10°C | | 0.834 | -42.0 | 95.9 | 29 |
| 6°C | | 0.808 | -37.8 | 94.6 | 19 |
| 2°C | | 0.849 | -40.6 | 96.4 | 29 |
| Acetylcholinesterase in the spinal cord | Log (Somite stage) | 98.3 | -134 | 93.7 | 37 |
| Acetylcholinesterase at neuromuscular junctions | Log (Somite stage) | 167 | -275 | 95.7 | 58 |
| Notochord vacuolisation- | Somite stage | | | | |
| 10°C | | 1.20 | -34.4 | 96.3 | 28 |
| 6°C | | 1.14 | -34.2 | 90.6 | 37 |
| 2°C | | 1.12 | -36.2 | 94.9 | 29 |

Temperature and maternal migratory groups have been combined, except for the lateral line primordium and notochord vacuolisation data, which are separated by temperature. $P<0.001$ for all regressions. [a] Embryos with more than 60 somites were classified according to their age, expressed as the number of somite-intervals after the beginning of somitogenesis. [b] Residual D.F. = Residual degrees of freedom, equal to n -2.

## Discussion

### *Muscle Development*

Myogenesis in trout follows patterns essentially similar to those previously described in zebrafish (*Brachydanio rerio*) (Waterman 1969) and herring (Vieira & Johnston 1992). Myoblasts within the somites form myotubes, which then produce myofibrils as they differentiate into visibly striated muscle fibres. The first muscle fibres formed develop from mononucleate (pioneer) myotubes, each produced by a single myoblast. Most myotubes, however, arise by fusion of a number of myoblasts, and thus most muscle fibres are multinucleate. Recent studies in zebrafish (Devoto *et al.* 1996) and pearlfish (*Rutilus frisii meidingeri* L.) (Stoiber *et al.* 1998) have expanded on this model, demonstrating that the pioneer myotubes are derived from a subset of a distinct group of adaxial cells located medially within the myotome, adjacent to the notochord. Most of these adaxial cells do not form pioneers, but rather migrate laterally to a more superficial position where they ultimately form the slow muscle fibres. The superficial zones of myogenesis described in this study, distinct from the more medial zone containing the muscle pioneers, presumably contain these differentiating slow muscle precursors.

### *Neural Development*

Axonogenesis of primary motor neurons occurs in a strict anterior-posterior sequence in trout; this differs from the irregular progression of axon formation described in zebrafish, in which axonogenesis at any given pair of somites may begin first at the more posterior somite (Eisen *et al.* 1986). The timing of axon emergence broadly coincides with the onset of HNK-1 immunoreactivity in the myosepta (Fig. 4.5), as also occurs in the zebrafish (Tongiorgi *et al.* 1995). The NCAM and L1 groups of molecules, all labelled by the HNK-1 antibody, are believed to play an important role in guidance of growth cones (Metcalfe *et al.* 1990) and establishment of neuromuscular junctions (Somasekhar & Nordlander 1995). If the primary motor neurons of trout are homologous to those of zebrafish (Eisen *et al.* 1986), then the concentration of HNK-1 immunoreactivity in the ventral region of the myosept may primarily function to guide the growth cone of the CaP primary motor neuron, which is the first to emerge from the spinal cord and innervates the ventral portion of the

myotome (Eisen *et al.* 1986). HNK-1 labelling is also concentrated to a slightly lesser extent in the dorsal region of each myosept; this expression may be involved in guidance of the growth cone of the MiP primary motor neuron, which emerges after the CaP and innervates the dorsal portion of the myotome (Eisen *et al.* 1986).

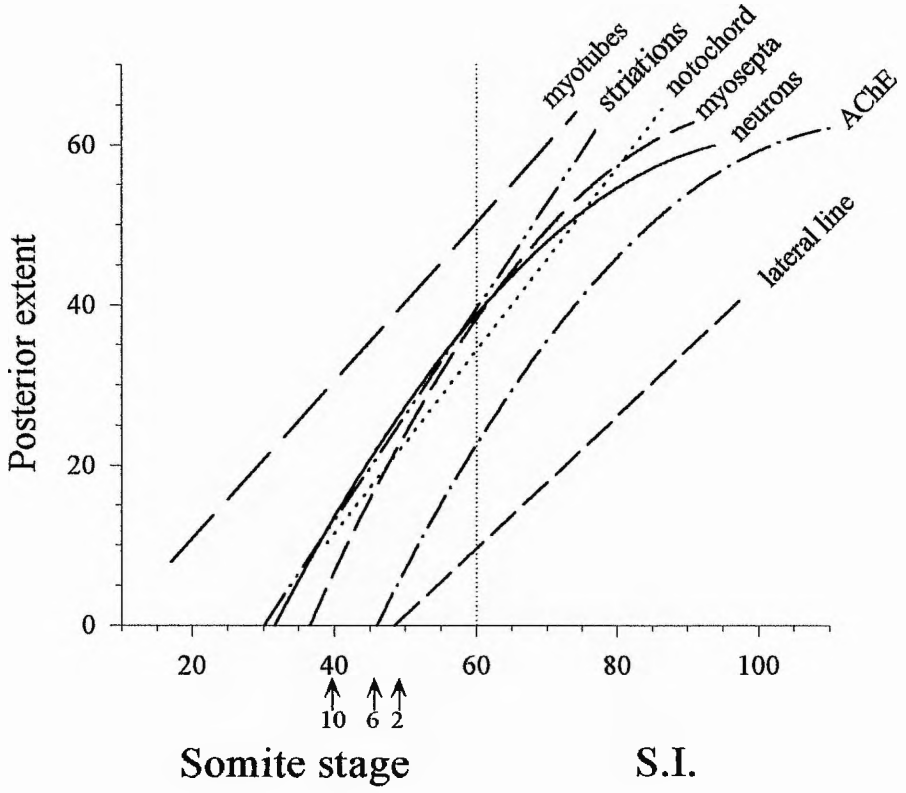



**Fig. 4.5.** Anterior-posterior progression, in terms of number of somites, of certain aspects of muscular and neural development. Data for the different temperatures and maternal migratory types have been combined. Linear regressions have been fitted for myotube formation and development of striations in the muscle fibres, notochord vacuolisation ('notochord') and the lateral line primordium. Second order regressions have been fitted for HNK-1 immunoreactivity at the myosepta ('myosepta'), primary motor neuron axonogenesis ('neurons') and acetylcholinesterase expression at myosepta ('AChE'). S.I. = Embryo age, expressed as the number of somite-intervals after the beginning of somitogenesis. The arrows beneath the x-axis indicate the stage at which spontaneous muscle contractions were first observed at 10°C, 6°C and 2°C (see Chapter 2, p. 97).

The fact that the leading tip of the migrating lateral line primordium was not labelled by the HNK-1 antibody suggests that pathfinding by the primordium is not mediated by L1 or NCAM molecules. The neural processes which will eventually innervate the neuromasts of the lateral line were labelled, however. It may be that the cells of the primordium serve as pathfinders for the extending neural processes, as has been suggested in zebrafish (Metcalfe 1985); at the same time, NCAM-mediated cell-cell adhesion may hold the numerous processes in their tightly-packed formation at the horizontal midline. The mechanism by which rearing at 10°C causes a delay in the posterior advancement of the primordium is unknown, but the consequences for the embryo are likely to be trivial; the lateral line must of very limited usefulness while the embryo remains inside the egg.

Rohon-Beard neurons are responsible for detecting touch stimulus during the embryonic phase (Clarke *et al.* 1984). The branching neural arbours, and the strong tendency for Rohon-Beard dendrites to be concentrated in fascicles between the tops of the somites, have also been described in *Xenopus* (Patton 1991). Rohon-Beard development in the Australian lungfish (*Neoceratodus forsteri* Krefft) differs somewhat from that in trout; in lungfish, Rohon-Beard dendrites initially all arch over the dorsal edges of the somites. Only later do these neural processes become concentrated at the dorsal intersomitic clefts (Whiting *et al.* 1992).

In trout, expression of acetylcholinesterase in the spinal cord precedes diffuse expression at the edges of the myotomes, which ultimately becomes concentrated at the neuromuscular endplates at the myosepta. Similar patterns have been described in herring (Johnston *et al.* 1995). As in trout, expression of acetylcholinesterase by herring primary motor neuron cell bodies coincides with the onset of axonogenesis.

***Regulation of Neuromuscular Development***

The process of vacuolisation of cells that will form the structural elements of the notochord proceeds similarly in trout as in zebrafish (Kimmel *et al.* 1995). As well as providing structural rigidity to the embryo, the notochord, in conjunction with the neural tube and floor plate, plays an important role in regulating

neuromuscular development. Diffusible factors produced by the notochord can promote myogenesis in unspecified somites (Buffinger & Stockdale 1994) and differentiation of motor neurons (Yamada *et al.* 1993); the notochord has also been shown to have a potentially inhibitory effect on the formation of muscle pioneers (Du *et al.* 1997), presumably to prevent premature muscle differentiation. The anterior-to-posterior progression of notochord vacuolisation coincides with development of striations in the muscle fibres and emergence of the primary motor neuron axons (Fig. 4.5), suggesting that the change in notochord structure may be correlated with alterations in its regulatory role. In quail, the importance of axial structures such as the notochord and neural tube diminishes once expression of myogenin and α-actin has begun in the myotubes (Pownall *et al.* 1996). The relatively late onset of notochord vacuolisation in embryos reared at lower temperatures does not, however, result in delays in muscle fibre formation or innervation. This suggests that any changes in the regulatory role of the notochord are not directly linked to vacuolisation, perhaps being completed by the time this structural change occurs.

Development of striations, motor neuron axonogenesis and notochord vacuolisation progress down the trunk of the embryo at similar somite stages. Expression of acetylcholinesterase in the spinal cord, and dendrite emergence of the Rohon-Beard dendrites also occur during this time, suggesting the onset of these events may be governed by similar processes. The rate of progression of development of striations remains regular to the last somite, however, while the other changes slow in their progress down the trunk as they approach the tail, suggesting at least two separate regulatory mechanisms (Fig. 4.5). Development of striations in the muscle fibres also progresses at a greater rate than myotube formation.

One of the final steps in formation of functional muscle is the synthesis of acetylcholinesterase (AChE) at the neuromuscular junctions. It has been reported that the onset of AChE synthesis is regulated directly by the motor neurons (Parkmatsumoto *et al.* 1992). This would explain the similarity in the shapes of the curves describing the progressions of motor neuron axonogenesis and AChE accumulation (Fig. 4.5).

As reported in Chapter 2, spontaneous twitches of the trunk muscle were observed in 40-somite trout embryos reared at 10°C (but not in embryos reared at 6° or 2°C), suggesting that muscular contractions can occur in the absence of synaptic AChE. The occurrence of myogenic contractions, occurring before the development of neural input, have been described in the Australian lungfish (Whiting *et al.* 1992) and have been suggested in the pearlfish (Stoiber *et al.* 1998). It has been shown in *Xenopus laevis* that the electrical stimulus for myogenic contractions can be transmitted from the most anterior somites to more posterior ones by cell-cell gap junctions at the inter-somitic boundaries (Blackshaw & Warner 1976); similar junctions have been found in embryos of tambaqui, (*Colossoma macropomum*) (Vieira & Johnston 1996). Early contractile activity may serve to regulate certain aspects of muscle development itself, such as migration of myonuclei to the periphery of the fibres, and formation of cross-striations (Parkmatsumoto *et al.* 1992, Ashby *et al.* 1993)

The effect of temperature on the timing of onset of first muscle contractions is clearly not mediated by changes in the timing of muscle fibre formation or innervation. In trout embryos, early neuromuscular developmental patterns are unaffected by either temperature or maternal migratory habit, and are highly canalised when compared to those of herring (Hill & Johnston 1997b; Johnston *et al.* 1997), in which the timing of muscle fibre formation and AChE synthesis can vary by the equivalent of 10 or more somite-intervals with a temperature difference of 10°C (Johnston *et al.* 1997). Temperature also affects the timings of onset of HNK-1 immunoreactivity and AChE synthesis at the myosepta in the angelfish (*Pterophyllum scalare* Lichtenstein) (Thomas 1998), and of formation of non-neuromuscular systems such as the gut, heart, pectoral fins, pronephric tubules and caudal fin in a variety of marine species (Fukuhara 1990; Johnston 1993; Gibson & Johnston 1995; Thomas 1998). Studies on salmonid species have reported minimal temperature effects on the relative timings of formation of organ systems (Vernier 1969; Ballard 1973c; Gorodilov 1989). The study described in Chapter 2 of the development of non-neuromuscular systems, such as the gut, pigmentation, fins and circulation, found no differences in development between offspring of different

maternal migratory types, and found no effects of temperature on the timing of organ formation.

Extreme temperature-induced changes in the developmental program may result in lethal abnormalities. The high level of canalisation in salmonid embryos may be associated with the greater duration of their embryonic phase relative to those of more rapidly-developing marine species, combined with the more variable thermal regimes which salmonid embryos normally experience in their shallow natal streams. The results suggest that trout have evolved regulatory mechanisms to ensure coordinated development at different temperatures.

# Chapter 5: Temperature and muscle growth in trout embryos and alevins

## Introduction

Growth rates during the first year of life are known to affect the timing of critical events such as smolting and reproductive maturation in Atlantic salmon (*Salmo salar*) (Thorpe 1989), and may partly determine migratory type in species with partial migration such as the trout (*S. trutta*) (Nordeng 1983). As most of the growth of a fish consists of increase in the mass of skeletal muscle, the dynamics of growth of this tissue are of particular interest - both in terms of total increase in muscle mass, and in the patterns of muscle production. The bulk of the myotome consists of 'white' fast-twitch muscle fibres, activated during fast-starts and at high cruising speeds. In contrast, the 'red' slow-twitch muscle fibres, which constitute a thin layer on the lateral surface of the myotome, are responsible for continuous swimming at slow-to-intermediate speeds (Bone 1966; Johnston *et al.* 1977; Jayne & Lauder 1994).

During the embryonic phase of growth in salmonids, recruitment of new fibres to the myotome is concentrated in germinal zones at the dorsal and ventral apices. Between hatching and yolk exhaustion, the pattern of fibre recruitment changes to that of adult fish; myosatellite cells produce new, post-embryonic fibres throughout the myotome, resulting in a 'mosaic' distribution of fibre sizes (Stickland *et al.* 1988; Johnston & McLay 1997). The myogenic cells responsible for embryonic and for post-embryonic muscle growth may belong to distinct populations (reviewed in Koumans & Akster 1995).

The number of fibres a fish recruits in order to achieve a given cross-sectional area of muscle can vary with growth rate (Higgins & Thorpe 1990; Kiessling *et al.* 1991; Alami-Durante *et al.* 1997), particularly in association with differences in temperature (Stickland *et al.* 1988; Vieira & Johnston 1992; Johnston 1993; Usher *et al.* 1994). The nature of such changes in muscle cellularity in response to temperature may also differ between white and red muscle (Johnston 1993; Johnston

*et al.* 1998), and between fish in the embryonic and in the post-embryonic phases of muscle growth (Johnston & McLay 1997).

Although muscle growth in juvenile and adult fish has generally been studied relative to body length (an indicator of total body growth), most studies on muscle growth of embryos and free embryos (alevins) have used sets of between-temperature comparisons at specific developmental stages. Nathanailides *et al.* (1995) and Johnston & McLay (1997), both studying *S. salar* embryos reared at different temperatures, made comparisons of muscle growth variables at the stage when 50% of embryos had hatched, and again at the estimated time of first feeding, when the yolk sac was near depletion. There is, however, evidence that the timing of both these stages, relative to the overall developmental sequence, can vary with environmental temperature (Heming 1982; Gorodilov 1983; Pavlov 1984; Rombough 1987; see also Chapters 2 & 3). Other studies on embryonic muscle growth in *S. salar* (Stickland *et al.* 1988; Usher *et al.* 1994; Matschak *et al.* 1995) have classified embryos according to the staging system described by Gorodilov (1983). However, the gaps between some of the later Gorodilov stages are equivalent to as much as 7.5% of the total development time, and the system has not been used to examine muscle growth during the period between hatching and first feeding, when the transition from embryonic to post-embryonic muscle growth occurs.

The aim of this study was to describe muscle growth patterns in trout reared at different temperatures, and with different maternal life histories, from embryos near the end of yolk sac vascularisation (step Y5) through to the onset of post-embryonic muscle growth. Muscle growth parameters were examined relative to total growth, as indicated by standard length, and relative to the degree of development, calculated using the scoring system for *S. trutta* described in Chapter 2. In addition, the effects of temperature on fibre numbers and diameters in first feeding fish of similar total muscle area were examined, and the numbers of post-embryonic fibres in such fish were quantified.

## Materials and Methods

### *Fish rearing and sampling*

The results presented in this chapter are from studies on fish produced from the 1995 fertilisation described in Chapter 2 (see p. 51-52; also see Appendix I). Samples were obtained from each of the ten maternal groups, which were subdivided among egg trays supplied with river water at two temperatures, 10°C (9.8-10.4°C) and 2°C (1.6-2.4°C). The migratory type of each female parent was determined from the profile of carotenoid pigments in the eggs, after Noack *et al.* (1996) (See Appendix II).

Pre-hatch samples were collected at 23 and 34 days post-fertilisation (d.p.f.) at 10°C, and at 90, 146 and 166 d.p.f. at 2°C. Samples were also taken at 50% hatching, which occurred at 43 and 192 d.p.f. at 10°C and 2°C respectively, and at the time of readiness to first feed (judged on the basis of the level of depletion of the yolksac), which occurred at 75 and 248 d.p.f. at 10°C and 2°C respectively. Individual samples consisted of 4 - 16 fish, taken from a range of maternal groups, but the offspring of anadromous and of freshwater resident female parents were examined separately. Embryos and alevins were killed by overdose of MS-222 (ethyl m-aminobenzoate) anaesthetic; between two and four specimens from each sample were fixed whole overnight in buffered 10% formalin (pH 7.2) and stored in 0.1% sodium azide in phosphate buffered saline (pH 7.2). The remaining specimens from each sample were fixed overnight in Bouin's fixative and stored in 70% ethanol; pre-hatch embryos were fixed whole, while alevins sampled at hatch and first feeding were fixed after removal of the head, tail and yolksac to improve penetration of the fixative.

### *Measurements*

Measurements of standard lengths of the hatch and first feeding specimens were made prior to fixation. Standard length measurements for pre-hatch samples were made from Bouin's-fixed specimens, and adjusted by a fixed-length-to-fresh-length correction factor, estimated on the basis of pre- and post-fixation measurements of formalin-fixed hatch samples. Differences in the level of shrinkage

of specimens between Bouin's and formalin fixation have been found to be negligible (M. Abercromby, pers. comm.) For each sample, mean developmental scores were calculated based on the formalin-fixed specimens, using the scoring system described in Chapter 2. Transverse wax sections (8μm) were cut at the anterior edge of the dorsal fin, stained with haematoxylin and eosin, and examined under a microscope with a drawing arm attachment. The outlines of all the muscle fibres, as demarcated by the basal lamina, on one side of each fish (red and white fibres examined separately) were traced using a drawing pen and pad linked to a Video-Plan Image Analysis System (Kontron Electronics, Basel), calibrated to calculate the cross-sectional area of each fibre traced. These values were converted to equivalent fibre diameters using the transformation:

$$\text{Diameter} = 2 \times (\text{Area}/\pi)^{0.5}$$

The mean diameters of the largest 200 white fibres and the largest 100 red fibres were calculated for each fish; changes in these variables indicate rates of fibre hypertrophy independently of fibre recruitment (Johnston *et al.* 1998). The nuclei in the white muscle on one side of each fish were counted.

At the time of first feeding, alevins had only recently entered the 'mosaic' phase of muscle growth, and so the first wave of post-embryonic fibres were still distinctly smaller than the surrounding embryonic fibres. However, apparently due to differences in the rates of fibre hypertrophy across the myotome, first-feeding alevins had fibres which were clearly post-embryonic, given their small size and location within the myotome, but which were larger than many of the most lateral embryonic fibres. Therefore, rather than using a maximum threshold size to identify post-embryonic fibres, any fibre less than one-quarter the cross-sectional area of each of the surrounding fibres was deemed to be a post-embryonic fibre. Numbers and sizes of such fibres were quantified from the drawings of the myotomes of the first feeding specimens.

Counts of total fibres, post-embryonic fibres and white muscle nuclei were doubled, on the assumption that the fish were bilaterally symmetrical. The muscle cellularity variables analysed and their abbreviated names are listed in Table 5.1.

**Table 5.1.** Abbreviations for the muscle cellularity variables studied.

| | Abbr. | Variable | Units |
|---|---|---|---|
| Length | $L$ | Standard length | mm |
| White muscle | $S_w$ | Sum of the white fibre cross-sectional areas | $mm^2$ |
| | $N_w$ | Number of white fibres per myotome | - |
| | $D_w$ | Mean white fibre diameter | μm |
| | $D_{L\text{-}w}$ | Mean diameter of the largest 200 white fibres | μm |
| | $N_{UC}$ | Number of nuclei per myotome in the white muscle | - |
| Red muscle | $S_r$ | Sum of the red fibre cross-sectional areas | $mm^2$ |
| | $N_r$ | Number of red fibres per myotome | - |
| | $D_r$ | Mean red fibre diameter | μm |
| | $D_{L\text{-}r}$ | Mean diameter of the largest 100 red fibres | μm |
| Post-embryonic fibres | $N_{pe}$ | Number of post-embryonic white fibres | - |
| | $D_{pe}$ | Mean diameter of post-embryonic white fibres | μm |

***Statistical analysis***

Using the Minitab statistical analysis package (Minitab Inc., U.S.A.), data on standard length at hatch and at first feeding were analysed by GLM (general linear model) ANOVA with temperature and maternal migratory type as main effects, followed by Tukey multiple comparison tests. Between-temperature comparisons, using 2-sample t-tests, were made of certain cellularity variables ($S_w$, $N_w$, $D_w$, $D_{L\text{-}w}$) between the 10°C hatch (43 d.p.f.) and 2°C late embryonic (166 d.p.f.) samples, which were very similar in terms of developmental score; between the 10°C hatch and 2°C mid-embryonic (146 d.p.f.) samples; and between the 10°C and 2°C first feeding samples.

The effects of temperature and maternal migratory type on the relationships between the variables listed in Table 5.1 were examined by multiple regression analysis. For each relationship between a variable $Y$ and a variable $X$, each specimen used was assigned a $T$ value ($T = 0$ for 2°C fish, $T = 1$ for 10°C fish) and an $M$ value

($M$ = 0 for offspring of anadromous females, $M$ = 1 for offspring of freshwater resident females). Using the Minitab software package, a regression was constructed for the relationship using factors such as $X$, $X^2$, $X^3$, $X^4$ for the general shape of the regression; $T$ and $M$ to test for possible simple additive effects of temperature and maternal migratory type; and $T$ x $X$, $T$ x $X^2$, $M$ x $X$ etc. to examine possible interactions between temperature / maternal migratory type and the $X$ variable. Factors whose individual significance values exceeded P=0.05 were successively omitted, until a regression model was arrived at for which all factors used had individual significance values of P<0.05, following the 'top-down' method described by Zar (1996). In certain cases, variables were log-transformed to reduce heterogeneity of variance.

For analysis of the effects of temperature on muscle cellularity at first feeding, only those alevins for which $S_w$ = 1.6 - 2.2 mm$^2$ were used. Between-temperature comparisons of $N_w$, $D_w$, $D_{L\text{-}w}$, $N_{UC}$, $N_{pe}$ and $D_{pe}$ were carried out using 2-sample t-tests. Numbers of specimens for which $S_w$ = 1.6 - 2.2 mm$^2$ were too small to allow meaningful comparisons between offspring of anadromous and of freshwater resident females.

## Results

### *Length and degree of development at hatch and at first feeding*

Newly-hatched fish reared at 2°C were, on average, over 3mm longer than newly-hatched fish reared at 10°C (P<0.001) (Fig. 5.1). By first feeding, the difference in lengths between the temperature groups had fallen to less than 1mm, but was still significant (P=0.004). At hatch, offspring of freshwater resident females were approximately 0.4mm longer than offspring of anadromous females (P=0.028), but maternal migratory type did not significantly affect length at first feeding. There were no significant interactions between temperature and maternal migratory type at either stage.

Examination of samples taken at 50% hatch, and at first feeding, at 10°C and 2°C revealed differences in the level of developmental advancement between temperature groups. Fish reared at 2°C hatched with greater numbers of rays in the

dorsal, anal and caudal fins, and with more advanced body pigmentation, implying that they emerged from the egg at a relatively later point in the developmental sequence than their 10°C counterparts (see also Chapter 2, p. 98). Newly-hatched 2°C alevins had a mean developmental score of 645 (range = 632 - 669), compared to 576 (range = 561 - 590) at 10°C. This difference of approximately 70 points is equivalent to 7% of the developmental sequence from fertilisation to first feeding.

When the samples taken at the estimated time of readiness to first feed were compared, the 2°C fish were less advanced than their 10°C counterparts, by approximately 50 points, having fewer segments at the dorsal fin rays, fewer procurrent caudal fin rays and more of the embryonic median finfold remaining. The mean score for first feeding fish was 906 (range = 879 - 926) at 2°C, and 955 (range = 937 - 975) at 10°C.

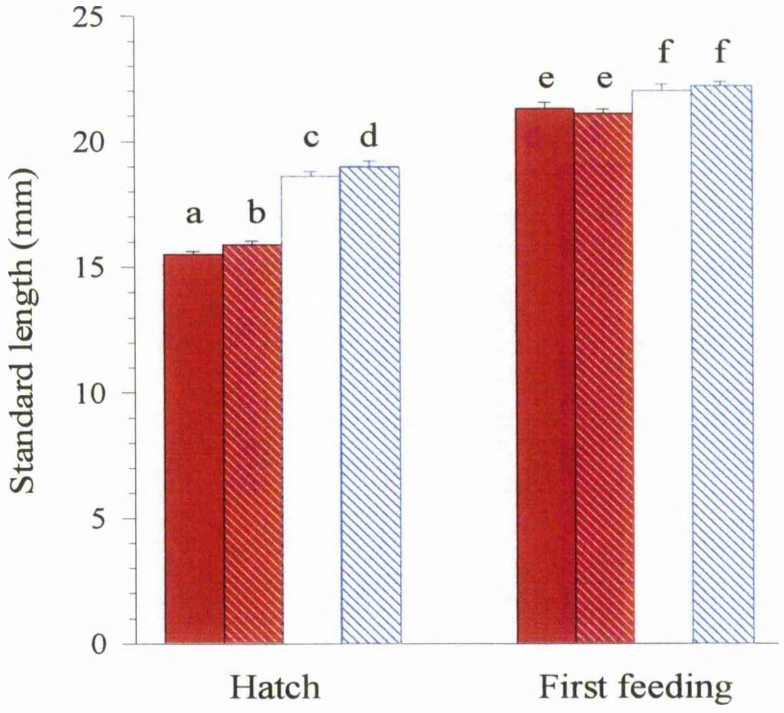



**Fig. 5.1.** Standard lengths at 50% hatch and at first feeding. Values are means ± S.E. 10°C, offspring of anadromous females: ■. 10°C, offspring of resident females: ▨. 2°C, offspring of anadromous females: □. 2°C, offspring of resident females: ▨. Significant differences between columns are indicated by the lack of a common lower-case letter.

***Muscle growth relative to development***

In order to relate total growth and, in particular, muscle growth, to developmental advancement at the different temperatures, plots of $L$, $S_w$, $N_w$ and $D_{L\text{-}w}$ against developmental score are presented in Figs. 5.2a-d. All four variables illustrate a trend for greater growth, relative to development, at the lower temperature (Figs. 5.2a-d). The 10°C hatch and 2°C late embryonic samples were at very similar levels of development (mean scores: 576 (10°C), 592 (2°C)); although the difference in total muscle area between these samples was not significant, the mean area of the largest 200 white fibres was significantly lower in the 10°C sample (Fig. 5.2d), and values of white fibre number differed even more substantially between the groups (Fig. 5.2c). Fish in the 10°C sample had over 1200 fewer fibres than fish in the 2°C sample.

The apparently greater between-temperature difference in relative levels of recruitment than in hypertrophy were supported by the comparison between the 10°C hatch sample and the 2°C mid-embryonic sample (mean score = 489). In spite of the fact that, in this comparison, the 10°C fish were more advanced, and had significantly higher values both of total muscle area, $S_w$ (P=0.018), and of mean area of the largest 200 white fibres, $D_{L\text{-}w}$ (P=0.001), they had significantly fewer white fibres (P=0.045), $N_w$ being approximately 90% those of the 2°C fish (Fig. 5.2c).

This relative reduction in fibre recruitment at 10°C, apparent by the time the 10°C fish hatched, was followed post-hatch by reductions in the relative rates of increase in length, total muscle area and muscle fibre size. In comparisons between the 10°C first feeding sample (mean score = 955) and the 2°C first feeding sample (mean score = 902), the 10°C group were again more advanced developmentally, but had now fallen behind, not only in terms of $N_w$ (P<0.001), but also in terms of $L$ (P=0.004), $S_w$ (P=0.001) and $D_{L\text{-}w}$ (P=0.001) (Figs. 5.2b-d).

Growth patterns in the red muscle were similar to those described for the white muscle, although the reduction in fibre recruitment at 10°C was relatively greater in the red muscle than in the white (not shown). Values of $N_r$ in the 10°C hatch sample were approximately 77% those of the 2°C late embryonic sample (P=0.015).

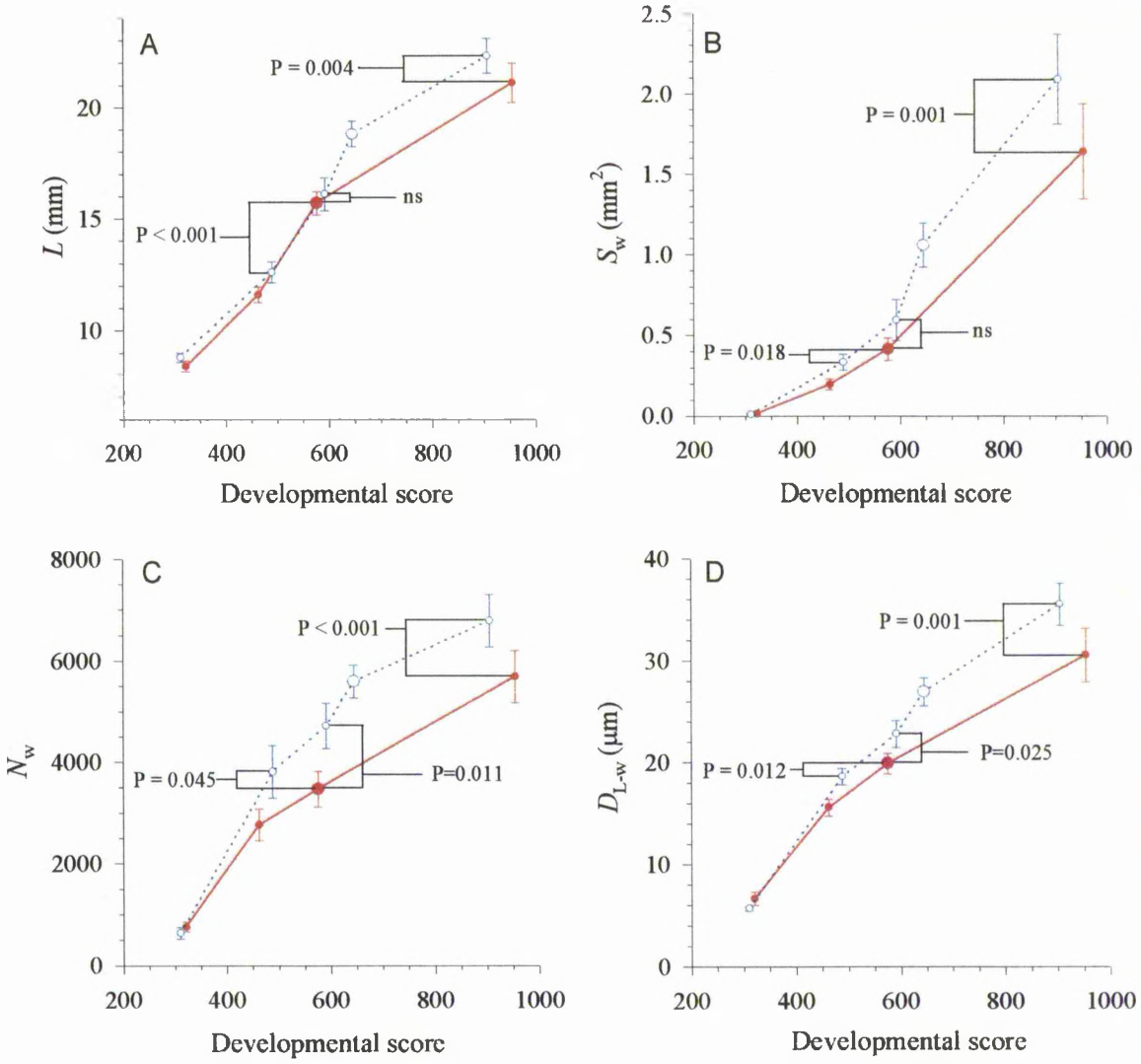



**Fig. 5.2.** Plots of certain growth variables against the mean developmental scores of equivalent sibling specimens. 10°C: Red, closed symbols, solid line. 2°C: Blue, open symbols, dotted line. Large symbols represent newly-hatched embryos. The P-values indicate significance of 2-sample t-tests between 2°C late embryo (n=7; score = 489) and 10°C hatch (n=11; score=576) samples, and between 2°C first feeding (n=13; score = 902) and 10°C first feeding (n=12; score = 955) samples. See Table 5.1 for explanation of abbreviations.

***Temperature and white muscle growth relative to length***

The various muscle cellularity variables were plotted against standard length, and in certain cases against each other; these relationships are shown in Figs. 5.3 - 5.4. Regression models are also illustrated in the graphs, and the equations listed in Tables 5.2 and 5.3. Where regression equations included significant temperature ($T$), $T$ x $X$-variable, migratory type ($M$), or $M$ x $X$-variable components, separate curves are shown as appropriate for temperatures and / or maternal migratory types.

Temperature affected the total area of white muscle, relative to length. The regression for the relationship between muscle area ($S_w$) (examined as Log($S_w$)) and $L$ included significant $T$, $T$ x $L$ and $T$ x $L^2$ components (Table 5.2). Differences in $S_w$ between temperature groups were trivial (<0.01mm$^2$) in 8-9mm fish (Fig. 5.3a), judged to be the minimum size at which the cross-sectional areas of the fibres could be accurately measured. However, towards the end of the embryonic period, muscle growth was relatively reduced at the higher temperature. At 15-17mm, 10°C embryos, which emerged from the egg at this size, had approximately 0.2mm$^2$ less white muscle than 2°C embryos of similar length; this difference amounts to 37% of $S_w$ in the 2°C embryos. Subsequently, the difference in $S_w$ between the temperature groups diminished, so that muscle area was again similar in both groups by 21-24mm, approximately the size range at which yolk supplies became exhausted and the fish were ready to begin feeding.

Temperature also affected rates of both muscle fibre recruitment and fibre hypertrophy. The regressions for white fibre number ($N_w$) against $L$ and for mean diameter of the largest 200 white fibres ($D_{L\text{-}w}$) against $L$ both included significant $T$, $T$ x $L$ and $T$ x $L^2$ components (Table 5.2), producing curves somewhat similar in shape to those for total muscle area. Embryos in the size range 15-17mm reared at 10°C had approximately 25% fewer white fibres than those reared at 2°C (Fig. 5.3b). By 21-24mm, this difference in fibre number between temperature groups was greatly reduced, but not eliminated.

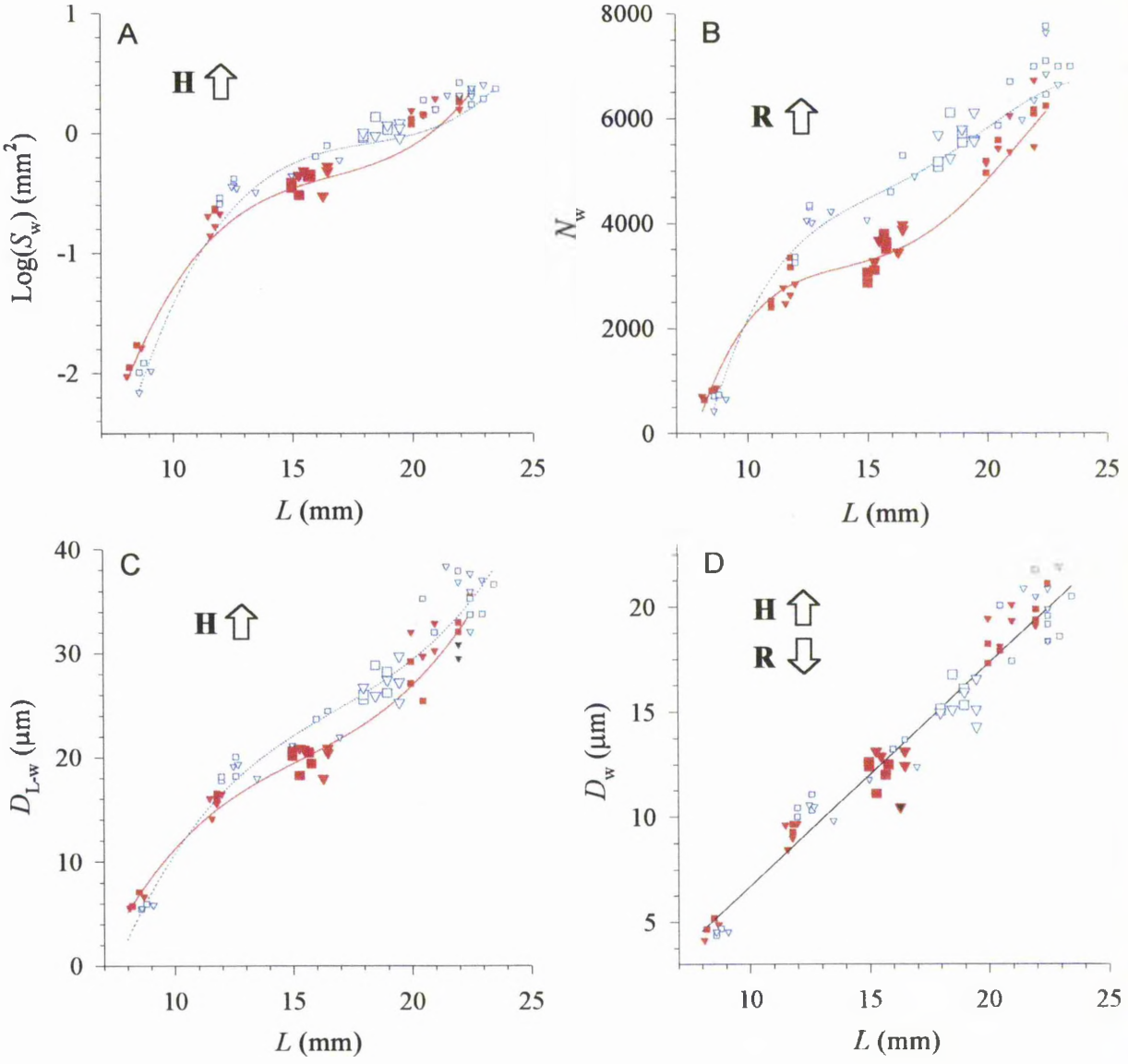



**Fig. 5.3.** Plots of white muscle cellularity variables against standard length. 10°C: Red, closed symbols, solid regression line. 2°C: blue, open symbols, dotted regression line. Squares and inverted triangles represent the offspring of anadromous and of freshwater resident females respectively. Large symbols represent newly-hatched embryos. The arrows indicate the theoretical directional effects on the Y-variable of relative increases in muscle fibre hypertrophy (H) or fibre recruitment (R). See Table 5.1 for explanation of abbreviations, Table 5.2 for the equations for fitted regressions.

The effect of temperature on $D_{L\text{-}w}$ was not as great in magnitude as that on $N_w$; at 15-17mm, the largest fibres of 10°C fish were approximately 13% smaller in diameter than those of 2°C fish of similar length (Fig. 5.3c). $D_{L\text{-}w}$ values around the time of first feeding were comparable between temperature groups.

Additional evidence for an effect of temperature on the relative rate of hypertrophy comes from the relationship between mean diameter of all the white fibres, $D_w$, and $L$ (Fig. 5.3d). $D_w$ is not an indicator of fibre hypertrophy alone, as it is affected by rates of recruitment of new, small fibres (Johnston *et al.* 1998). The relationship between $D_w$ and $L$ was not significantly affected by temperature. Therefore it can be inferred that, at 2°C, any relative reduction in $D_w$ resulting from the higher rates of fibre recruitment must have been offset by higher rates of fibre hypertrophy.

The relationships between fibre number and length, and between mean fibre diameter and length, were similar for the white and red muscle. However, temperature did not significantly affect the relationship between $D_{L\text{-}r}$ (the mean diameter of the largest 100 red fibres) and length.

***Temperature effects on fibre recruitment and fibre hypertrophy***

The between-temperature differences in the relationships depicted in Fig. 5.4a-d are all consistent with a relatively greater rate of recruitment of muscle fibres at 2°C, and relatively greater fibre hypertrophy at 10°C. Fish with a given value of total muscle area ($S_w$) had significantly higher fibre numbers (Fig. 5.4a), but lower mean fibre diameters when reared at 2°C than when reared at 10°C (Fig. 5.4b). Also, relative to white fibre number, $N_w$, fish reared at 10°C had larger fibres (indicated both by $D_w$ and by $D_{L\text{-}w}$) than fish reared at 2°C (Fig. 5.4c-d). However, the relationship between $D_{L\text{-}w}$ and $S_w$ was not significantly affected by temperature (Fig. 5.4e).

In the red muscle, temperature did not significantly affect the relationship between $D_{L\text{-}r}$ and $N_r$. Otherwise, the relationships amongst the various muscle cellularity variables were similar for the white and red muscle.

***Temperature and the number of nuclei in the white muscle***

The effect of temperature on the number of nuclei per myotomal cross-sectional area in the white muscle ($N_{UC}$) was broadly similar to that of temperature on white fibre number, $N_w$. Fish in the 15-17mm size range had approximately 40% fewer nuclei when reared at 10°C than when reared at 2°C (not shown). This difference was greatly reduced by 21-24mm. Reflecting the similar responses of $N_{UC}$ and $N_w$ to temperature, the relationship between $N_{UC}$ and $N_w$ was not significantly different between 10°C and 2°C (not shown).

***Relative temperature effects on red and white muscle growth***

Muscle growth progressed more rapidly in the white than in the red muscle. In fish with 0.5mm$^2$ of white muscle (i.e. fish of approximately 15-17mm standard length), the ratio of white muscle area : red muscle area was approximately 25:1. In fish with 2mm$^2$ of white muscle (around the first feeding stage), this ratio had increased to approximately 43:1. This was due to greater rates both of fibre recruitment and fibre hypertrophy in the white muscle.

Temperature did not significantly alter the relative proportions of red and white total muscle area, or the relationships between $D_r$ and $D_w$, or between $D_{L\text{-}r}$ and $D_{L\text{-}w}$ (Table 5.3). However, temperature did significantly affect the relationship between $N_r$ and $N_w$ (P=0.009) - fish reared at 2°C had significantly more red fibres, relative to the number of white fibres, than those reared at 10°C (Table 5.3), although this difference amounted to only 16 extra red fibres at the lower temperature.

***Effects of maternal migratory type***

Most of the muscle cellularity variables studied were not found to be affected by the migratory type of the female parent. The only significant effects detected were in the relationships involving the mean diameter of the 100 largest red fibres ($D_{L\text{-}r}$). The offspring of anadromous females had significantly greater values of $D_{L\text{-}r}$ relative to $L$, $N_r$, $S_r$ and $D_{L\text{-}w}$ (Table 5.3, Fig. 5.4f).

***Temperature and white muscle cellularity at first feeding***

Values of total white muscle area, $S_w$, overlapped somewhat between first feeding groups from the two temperatures. Between-temperature comparisons of muscle cellularity variables made using only the fish falling within this region of overlap (i.e. those fish for which $S_w$ = 1.6 - 2.2mm$^2$) found no significant effects of temperature on any of the white muscle cellularity variables ($S_w$, $N_w$, $D_w$, $D_{L\text{-}w}$, $N_{UC}$) (Table 5.4).

At the time of first feeding, post-embryonic fibres were clearly distinguishable from the surrounding, larger embryonic fibres (Fig. 5.5). The criterion for assignment of 'post-embryonic' status to a fibre was that its cross-sectional area should not exceed 25% of that of any of the adjacent fibres. (Fibres fitting this criterion were not found in fish sampled prior to first feeding.) Greater numbers of post-embryonic fibres were normally found in the epaxial (dorsal) quadrants of the myotome than in the hypaxial (ventral) quadrants. Temperature significantly affected the numbers of post-embryonic fibres present in fish for which $S_w$ = 1.6 - 2.2mm$^2$; those reared at 10°C had over twice as many post-embryonic fibres compared to those reared at 2°C (P=0.0067; Table 5.4). Temperature did not significantly affect the mean diameter of the post-embryonic fibres ($D_{pe}$). Histograms illustrating diameter frequencies of the post-embryonic fibres are presented in Fig. 5.6.

**Fig. 5.4.** Plots of certain muscle cellularity variables against $S_w$, against $N_w$, and against $S_r$. 10°C: Red, closed symbols, solid regression line. 2°C: Blue, open symbols, dotted regression line. Squares and inverted triangles represent the offspring of anadromous and of freshwater resident females respectively. Large symbols represent newly-hatched embryos. In **F**, temperature had no significant effect on the relationship; the bold and normal lines represent the regressions for offspring of anadromous and of freshwater resident females respectively. The arrows indicate the theoretical directional effects on the Y-variable of muscle fibre hypertrophy (H) or fibre recruitment (R). See Table 5.1 for explanation of abbreviations, Tables 5.2 and 5.3 for the equations for fitted regressions.

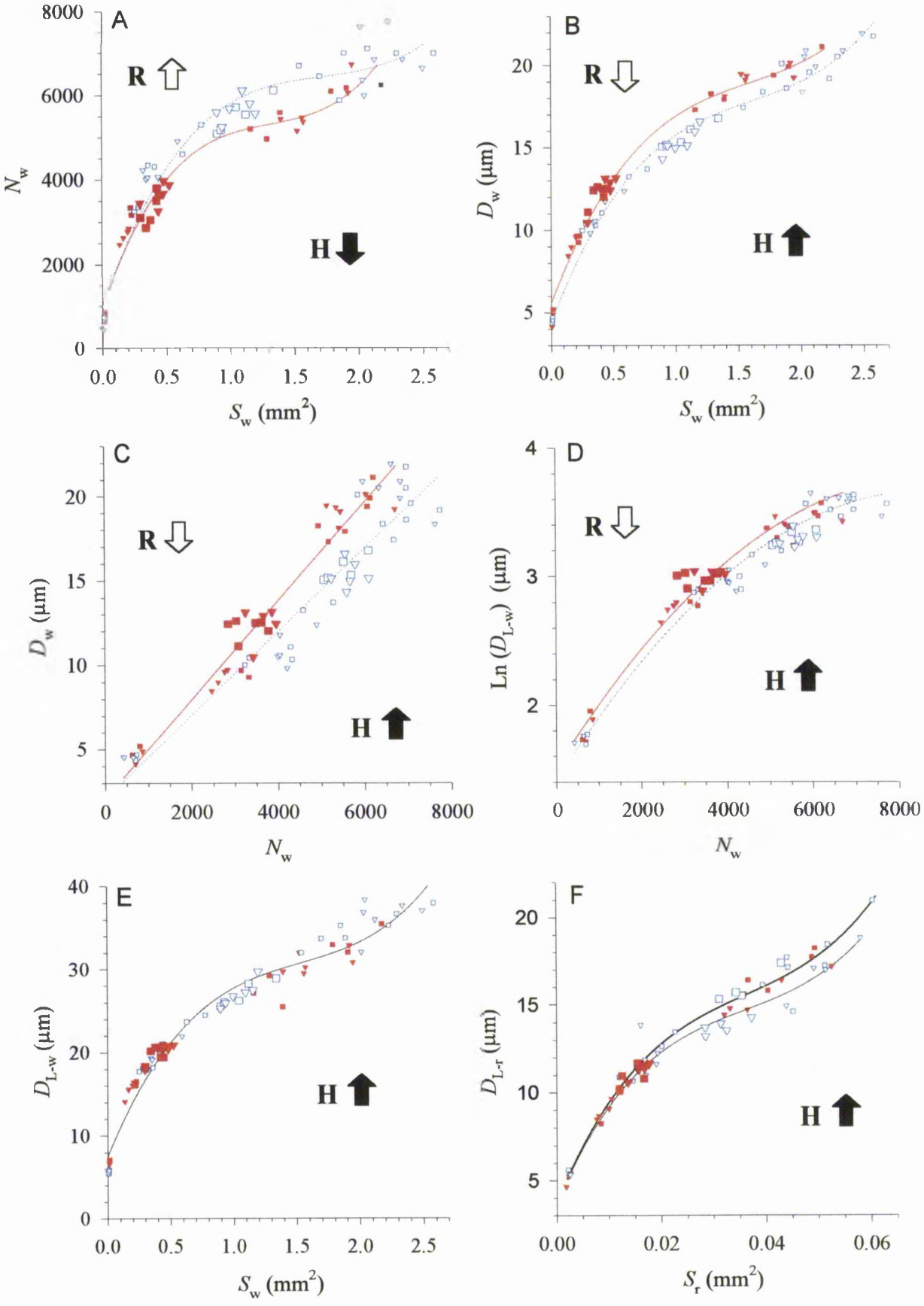

A
R
H
$N_w$
$S_w$ (mm$^2$)
B
R
H
$D_w$ (µm)
$S_w$ (mm$^2$)
C
R
H
$D_w$ (µm)
$N_w$
D
R
H
Ln ($D_{L-w}$) (µm)
$N_w$
E
H
$D_{L-w}$ (µm)
$S_w$ (mm$^2$)
F
H
$D_{L-r}$ (µm)
$S_r$ (mm$^2$)

**Table 5.2.** Regression equations for white muscle cellularity variables against $L$, against $S_w$ and against $N_w$.

| Y | X | Equation | $r^2_{adj}$ | Individual P values | D.F. | Shown in Fig.: |
|---|---|---|---|---|---|---|
| $S_w$ | $L$ | * Log($S_w$) = -12.5 + 1.98($L$) - 0.107($L^2$) + 0.00196($L^3$) + **0.218($T$) - 0.295($T$x$L$) + 0.00907($T$x$L^2$)** | 97.8 | All <0.001 | 6, 66 | 5.3a |
| $N_w$ | $L$ | $N_w$ = -43209 + 10592($L$) - 875($L^2$) + 34.4($L^3$) - 0.484($L^4$) + **6215($T$) - 892($T$x$L$) - 26.6($T$x$L^2$)** | 96.9 | All <0.001, except for $L^3$ (P=0.001) and $L^4$ (P=0.002) | 7, 67 | 5.3b |
| $D_{L\text{-}w}$ | $L$ | $D_{L\text{-}w}$ = -70.5 + 14.4($L$) - 0.779($L^2$) + 0.0155($L^3$) + **16.5($T$) - 2.26($T$x$L$) + 0.0656($T$x$L^2$)** | 95.4 | All <0.001, except for $T$ (P=0.012), $T$x$L$ (P=0.010) and $T$x$L^2$ (P=0.017) | 6, 65 | 5.3c |
| $D_w$ | $L$ | $D_w$ = -3.90 + 1.06($L$) | 95.2 | Both <0.001 | 1, 71 | 5.3d |
| $N_w$ | $S_w$ | $N_w$ = 973 + 8733($S_w$) - 4850.1($S_w^2$) + 940.7($S_w^3$) - **1355($T$x$S_w^2$) + 634($T$x $S_w^3$)** | 94.8 | All <0.001 | 5, 67 | 5.4a |
| $D_w$ | $S_w$ | $D_w$ = 4.01 + 24.6($S_w$) - 20.5($S_w^2$) + 8.69($S_w^3$) - 1.32($S_w^4$) + **1.04($T$)** | 98.8 | All <0.001 | 5, 67 | 5.4b |
| $D_w$ | $N_w$ | $D_w$ = 2.07 + 0.00293($N_w$) + **0.000455($T$x$N_w$)** | 91.1 | All <0.001 | 3, 68 | 5.4c |
| $D_{L\text{-}w}$ | $N_w$ | * Ln($D_{L\text{-}w}$) = 1.42 + 0.000520($N_w$) - 0.00000003($N_w^2$) + **0.0967($T$)** | 96.4 | All <0.001 | 2, 72 | 5.4d |
| $D_{L\text{-}w}$ | $S_w$ | $D_{L\text{-}w}$ = 7.48 + 36.7($S_w$) - 21.1($S_w^2$) + 4.60($S_w^3$) | 96.4 | All <0.001 | 3, 68 | 5.4e |
| $N_{UC}$ | L | * Ln($N_{UC}$) = -3.52 + 1.75($L$) - 0.0899($L^2$) + 0.00155($L^3$) + **0.912($T$) - 0.0137($T$x$L^2$) + 0.000527($T$x$L^3$)** | 95.2 | All <0.001, except for constant (P=0.001) | 6, 65 | - |
| $N_{UC}$ | $S_w$ | * Ln($N_{UC}$) = 6.03 + 6.51($S_w$) - 6.84($S_w^2$) + 3.06($S_w^3$) - 0.480($S_w^4$) - **0.0884($T$x$S_w$)** | 96.4 | All <0.001, except for $T$x$S_w$ (P=0.011) | 5, 64 | - |
| $N_{UC}$ | $N_w$ | * Ln($N_{UC}$) = 5.587 + 0.000698($N_w$) - 0.00000004 ($N_w^2$) | 95.5 | All <0.001 | 2, 69 | - |

*Equations using Log(Variable) or Ln(Variable) to reduce heterogeneity of variance. Bold print indicates equation components involving temperature or maternal migratory type. D.F. = Degrees of freedom associated with the regression and with the error. See Table 5.1 for explanation of abbreviations. All regressions had significance values of P<0.001.

**Table 5.3.** Regression equations for red muscle cellularity variables against standard length, against sum of the red fibre areas, against red fibre number, and against white muscle cellularity variables.

| Y | X | Equation | $r^2_{adj}$ | Individual P values | D.F. |
|---|---|---|---|---|---|
| $S_r$ | $L$ | $S_r = -0.00439 + 0.000107(L^2)$ - **0.000225(*T*x*L*)** | 91.7 | 0.002 (Constant), <0.001 ($L^2$), 0.001 (*T*x*L*) | 2, 68 |
| $N_r$ | $L$ | $N_r = -2994 + 832(L) - 79.0(L^2) + 3.286(L^3) - 0.04985(L^4)$ + **288(*T*)** - **44.3(*T*x*L*)** + **1.36(*T*x$L^2$)** | 93.7 | All <0.001 | 7, 66 |
| $D_r$ | $L$ | $D_r = -2.76 + 1.05(L) - 0.0159(L^2)$ | 88.7 | 0.047 (Constant), <0.001 ($L$), 0.006 ($L^2$) | 2, 70 |
| $D_{L\text{-}r}$ | $L$ | $D_{L\text{-}r} = 0.785(L)$ - **0.865(*M*)** | 85.2 | <0.001 ($L$), 0.018 ($M$) | 2, 62 |
| $N_r$ | $S_r$ | $N_r = 137 + 9130(S_r) - 87200\ (S_r^2)$ - **27.2(*T*)** | 91.1 | All <0.001 | 3, 67 |
| $D_r$ | $S_r$ | $D_r = 3.38 + 504\ (S_r) - 11600\ (S_r^2) + 106000\ (S_r^3)$ + **0.568(*T*)** | 97.0 | All <0.001 | 4, 66 |
| $D_{L\text{-}r}$ | $S_r$ | $D_{L\text{-}r} = 3.74 + 711(S_r) - 16000\ (S_r^2) + 148000\ (S_r^3)$ - **22.9(*M*x$S_r$)** | 97.3 | All <0.001 ***(See Fig. 5.4f)*** | 4, 59 |
| $D_r$ | $N_r$ | $D_r = 0.0414(N_r) - 0.000024(N_r^2)$ + **1.02(*T*)** | 74.7 | <0.001 ($N_r$), 0.024 ($N_r^2$), 0.005 ($T$) | 3, 69 |
| $D_{L\text{-}r}$ | $N_r$ | $D_{L\text{-}r} = 0.0460(N_r)$ - **0.00402(*M*x$N_r$)** | 79.7 | <0.001 ($N_r$), 0.009 ($M$x$N_r$) | 3, 61 |
| $S_r$ | $S_w$ | * $S_r = 0.0318 + 0.0192\ (\text{Ln}(S_w)) + 0.00282(\text{Ln}(S_w))^2$ | 92.9 | All <0.001 | 2, 68 |
| $N_r$ | $N_w$ | $N_r = 112 + 0.0391(N_w)$ - **16.0(*T*)** | 91.5 | <0.001 (Constant), <0.001 ($N_w$), 0.009 (T) | 2, 71 |
| $D_r$ | $D_w$ | $D_r = 1.24 + 0.816(D_w) - 0.0118(D_w^2)$ | 92.0 | 0.035 (Constant), <0.001 ($D_w$), 0.001 ($D_w^2$) | 2, 70 |
| $D_{L\text{-}r}$ | $D_{L\text{-}w}$ | $D_{L\text{-}r} = 4.59 + 0.0273(D_{L\text{-}w}^2)$ - $0.000482(D_{L\text{-}w}^3)$ - **0.0287(*M*x$D_{L\text{-}w}$)** | 90.0 | All <0.001, except for $M$x$D_{L\text{-}w}$ component (P=0.018) | 3, 60 |

*Equations using Ln(Variable) to reduce heterogeneity of variance. Bold print indicates equation components involving temperature or maternal migratory type. D.F. = Degrees of freedom associated with the regression and with the error. See Table 5.1 for explanation of abbreviations. All regressions had significance values of P<0.001.

**Table 5.4.** Comparisons of white muscle cellularity variables, including post-embryonic fibre variables, in first-feeding fish of comparable muscle cross-sectional area ($S_w$ = 1.6 - 2.2mm$^2$).

| Variable | 10°C | 2°C | P-value | D.F. |
|---|---|---|---|---|
| $S_w$ ($mm_2$) | 1.950 ± 0.063 (5) | 1.953 ± 0.055 (7) | 0.97 | 11 |
| $N_w$ | 6242 ± 122 (5) | 6502 ± 224 (7) | 0.33 | 11 |
| $D_w$ (μm) | 19.94 ± 0.33 (5) | 19.52 ± 0.41 (7) | 0.45 | 11 |
| $D_{L\text{-}w}$ (μm) | 32.8 ± 0.76 (5) | 35.12 ± 0.81 (7) | 0.067 | 11 |
| $N_{UC}$ | 4405 ± 343 (5) | 5010 ± 252 (6) | 0.20 | 10 |
| $N_{pe}$ | 419 ± 55 (5) | 132 ± 5.8 (5) | 0.0067 | 9 |
| $D_{pe}$ (μm) | 7.00 ± 0.28 (5) | 7.01 ± 0.26 (5) | 0.99 | 9 |

Values are means ± S.E.; numbers of fish per group indicated in brackets. D.F. = Degrees of freedom. See Table 5.1 for explanation of abbreviations.

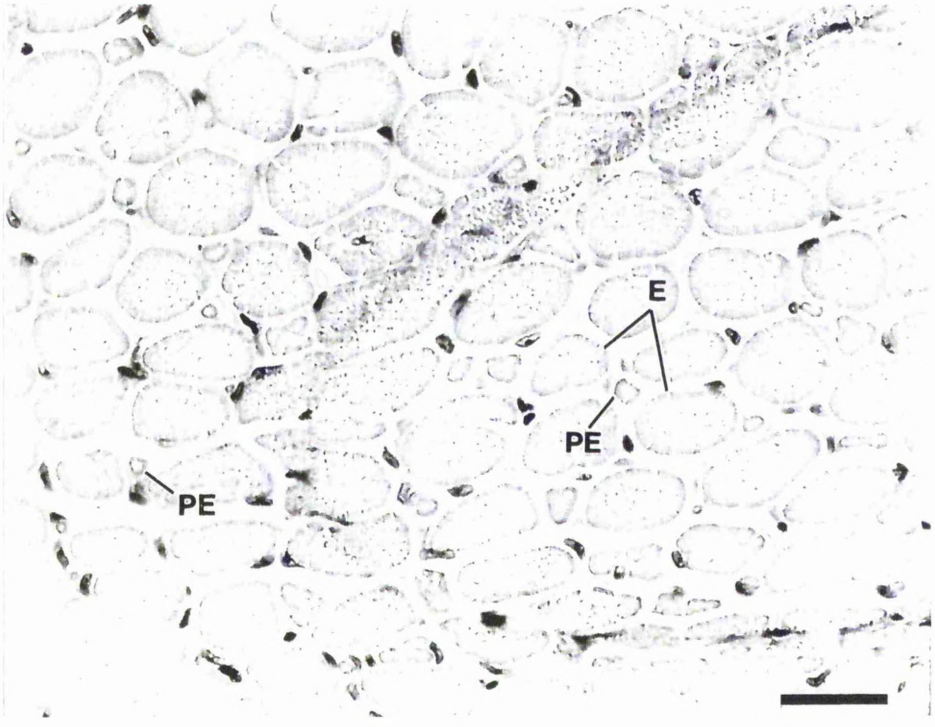



**Fig. 5.5.** Transverse wax section through the epaxial white muscle of a 10°C first-feeding fish, stained with haematoxylin and eosin. E = embryonic fibre; PE = post-embryonic fibre. Scale bar = 25μm.

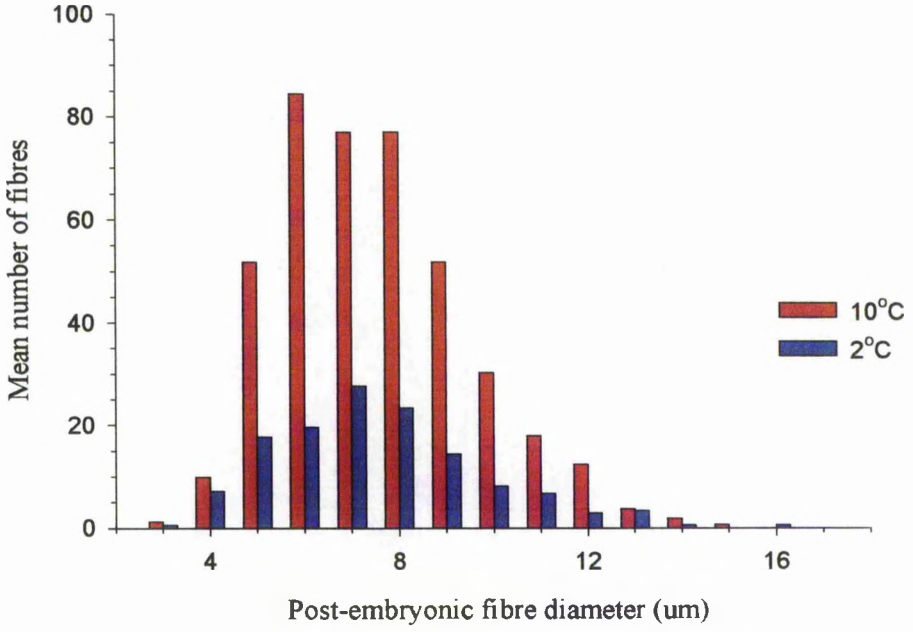



**Fig. 5.6.** Frequency histograms for the diameters of the post-embryonic muscle fibres. Values are means (n=5 at each temperature) for first-feeding fish of comparable muscle cross-sectional area ($S_w$ = 1.6 - 2.2mm$^2$).

## Discussion

***Temperature affected length at hatch and at first feeding***

Newly-hatched fish were shorter when reared at 10°C than when reared at 2°C. This was probably due to the less advanced developmental state on hatching at 10°C, as indicated by the developmental scores. Precocial hatching in salmonid embryos reared at elevated temperatures has been reported previously (Heming 1982; Gorodilov 1983; Pavlov 1984, see also Chapter 2); it has been speculated that embryos reared at high temperatures may need to emerge from the egg relatively earlier in order to avoid hypoxia (Matschak *et al.* 1995, see also Chapter 2). The 2°C fish were still longer than their 10°C counterparts at first feeding, however, despite having lower developmental scores.

This implies that fish reared at lower temperatures are longer, relative to their developmental state, as has been reported by numerous authors (Peterson *et al.* 1977; Beacham & Murray 1985; Johnston *et al.* 1997), and which is in agreement with the findings reported in Chapter 3 (which related to fish from the 1996 fertilisation). The less advanced state of the 2°C fish when sampled at 'first feeding' (i.e. near yolk exhaustion), compared to the 10°C fish, may be indicative of slower development relative to yolk utilisation at the lower temperature, again in agreement with the findings of Chapter 3. However, the quantity of remaining yolk was not measured in specimens collected for this study, and it is important to bear in mind the somewhat imprecise basis on which the timing of sampling at first feeding was decided, one of visual inspection of alevins in the hatchery tanks.

***Temperature affected total muscle growth***

Towards the end of the embryonic period, fish reared at 10°C had reduced total muscle growth when compared to fish reared at 2°C, relative both to developmental score and to length. By first feeding, the differences in $S_w$ between temperatures were no longer evident when examined relative to length, but persisted when examined relative to the degree of development. This is consistent with the finding in Chapter 3 that temperature differentially affects the processes of development, increase in length and increase in mass, and that, at least in the case of length, such effects differ between embryonic and post-hatch periods.

Stickland *et al.* (1988), studying embryonic muscle growth in Atlantic salmon, found no significant effects of temperature on total muscle area, relative to the developmental stages of Gorodilov (1983). The relationship between muscle growth and total growth was not described. More recent studies have reported that rearing of Atlantic salmon embryos at higher temperatures results in reductions in both fibre number and in mean fibre size around the time of hatching (Johnston & McLay 1997; Matschak *et al.* 1997), probably linked to similar reductions in the levels of the cellular myc transcript (associated with cell division) and of mRNA for the myofibrillar proteins actin and MyHC, as described by Matschak & Stickland (1996). Muscle fibre number and size are also reduced at high temperatures in the rainbow trout (*Oncorhynchus mykiss*) (Matschak *et al.* 1998). The findings of

Johnston & McLay (1997) were based on comparisons at the stages of 50% hatch and first feeding, but the degree of development of the newly-hatched embryos and first feeding alevins was not ascertained. The reductions in muscle growth in newly-hatched embryos reared at higher temperatures, although entirely consistent with the findings of the present study, may nonetheless have been partly due to a precocious onset of hatching.

***Temperature differentially affected fibre recruitment and fibre hypertrophy***

Although levels of both fibre recruitment and fibre hypertrophy were reduced at 10°C, overall the findings indicate that fibre recruitment was affected to a relatively greater extent than hypertrophy. As a result, relative to total muscle area, a trend towards fewer but larger fibres was observed in fish reared at 10°C. By first feeding, the post-hatch 'catch-up' in recruitment may, however, have led to some reduction in the between-temperature differences in muscle cellularity, at least sufficiently to explain the lack of significance of the tests carried out on the first-feeding subgroups. The small sample sizes of these subgroups may also have contributed to the lack of significant differences.

In certain cases, embryos of Atlantic salmon have been found to have reduced fibre numbers but increased mean myofibrillar areas when reared at a higher temperature, so that total muscle area is unaffected (Stickland *et al.* 1988; Usher *et al.* 1994; Nathanailides *et al.* 1995). In these studies, only a small subset (approximately 100-200) of the white fibres, located in the upper epaxial region of the myotome, was used for size measurements. The accuracy with which the fibres from this region represent the entire white muscle could be expected to vary with changes in muscle growth patterns, particularly as this region excludes the germinal zones but includes the area of greatest post-embryonic fibre production in first feeding alevins. Also, values of fibre number and mean fibre size were not normalised against body length or total muscle area. Nevertheless, the trend towards reduced fibre numbers relative to fibre size in salmon embryos reared at high temperatures is in agreement with the results of this study.

***The post-hatch 'catch-up' in fibre recruitment at 10°C***

First-feeding fish of comparable total muscle area ($S_w$ = 1.6 - 2.2mm$^2$) had greater numbers of post-embryonic fibres at 10°C than at 2°C. If this higher rate of post-embryonic fibre production continues at the higher temperature, then the relative reduction in fibre recruitment at 10°C during the early life stages may ultimately be compensated for after first feeding. It is important to note that after the fish reared at 10°C hatched, the between-temperature difference in white fibre number at a given length went from being approximately 1200 at 16mm to just 500 at 22mm – indicating a relative 'catch-up' of approximately 700 fibres at 10°C. As the difference between temperatures in post-embryonic fibre number at first feeding was only approximately 300 (Table 5.4), the catch-up in white fibre number at 10°C was not solely due to higher recruitment of the post-embryonic fibres. There must also have been greater numbers of embryonic fibres produced at 10°C between 16 and 22mm. The greater numbers of post-embryonic fibres at 10°C than at 2°C therefore probably reflects an overall increase in recruitment after hatching, rather than differing responses to temperature of embryonic and post-embryonic myoblasts.

***Possible mechanisms and consequences of temperature effects on muscle growth***

The reduction in pre-hatch muscle growth at the higher temperature may be due to a limiting effect of the chorion on oxygen concentration inside the egg. Hatching has been shown to be associated with a sharp drop in the critical oxygen concentration requirements of salmonid embryos (Rombough 1987); if levels of oxygen become a limiting factor at high temperatures, this could explain not only the relatively earlier hatching at 10°C, but also the reduction in total muscle growth. *In vitro* studies have shown that conditions of limited resource availability favour cell growth relative to cell division (Cheek & Hill 1970), perhaps because the protein synthesis required for growth may be a more efficient method of increasing tissue mass than the nuclear replication required for production of new cells. Oxygen deficiency at 10°C could therefore also be the reason for the shift in muscle growth in favour of hypertrophy, rather than the more metabolically-demanding recruitment of new fibres. It has been shown that the effects of temperature on muscle cellularity in salmon embryos are altered by changes in oxygen concentration or removal of the embryos from the chorion (Matschak *et al.* 1995; 1997; 1998).

Interestingly, in the present study, hypertrophy of fibres in the red muscle did not appear to be affected by temperature. The red fibres should theoretically be less vulnerable to the low oxygen levels at high temperatures, given their greater surface area : volume ratios and their proximity to the surface of the embryo. However, recruitment of fibres was more severely reduced at the higher temperature in the red muscle than in the white. Temperature may modulate patterns of muscle growth by mechanisms other than limitation of resources. In Chapter 3, it was found that conversion of yolk into body mass was achieved more efficiently during the embryonic period at 10°C than at 2°C, and this may be partly due to a reduction in levels of cellular division in favour of cell growth.

Reductions in total muscle area at hatching are unlikely to have serious deleterious consequences for the survival of trout embryos, as they spend most of the ensuing endogenous feeding phase buried in the gravel of the streambed. Differences in the quantity of muscle present at first feeding, however, such as were found relative to developmental score, are likely to have an impact on the ability of newly-emerged alevins to find food, avoid predators and locate suitable territories. An increase in water temperature may thus have negative consequences for trout survival during the early stages of life in the stream; however, this might be countered by long-term benefits arising from greater rates of production of post-embryonic muscle fibres during the first year. In Arctic charr (*Salvelinus alpinus*), improved growth rates are associated with an increase in the proportion of freshwater residents (Nordeng 1983), suggesting that environment-mediated changes in growth opportunity may influence life history strategy. In trout there is also evidence of a genetic contribution to migratory type, operative through the female parent (Clarke *et al.* 1994; Thompson 1995). In the experiment described in this chapter, however, the migratory type of the female parent was found to have very little effect on growth prior to first feeding.

# Chapter 6: The effects of exercise and embryonic thermal history on muscle growth in juvenile trout

## Introduction

The temperature which a fish experiences during the embryonic and larval periods can have long-term consequences for patterns of muscle growth. Johnston *et al.* (1998) reared spring-spawned herring (*Clupea harengus*) embryos at 5°C and 8°C; when each group reached first feeding, they were transferred to a common, ambient temperature. The larvae which had been reared at the higher temperature as embryos exhibited significantly higher growth rates after first feeding than those reared at the lower temperature, although they recruited fewer muscle fibres to achieve a given muscle mass. During larval and adult life, both muscle fibre growth and new fibre formation are dependent on the production of additional muscle nuclei by myosatellite cells (Moss & LeBlond 1971; Snow 1983; Powell *et al.* 1989; Koumans *et al.* 1990; Johnston *et al.* 1998). Johnston *et al.* (1998) suggested that the faster growth of the 8°C-reared herring might be associated with the higher numbers of myosatellite cells found in newly-hatched embryos reared at 8°C when compared with those reared at 5°C (Johnston 1993).

Rates of growth can also be improved when fish are subjected to regimes of forced swimming exercise. Houlihan & Laurent (1987) found that growth rates in rainbow trout (*Salmo gairdneri*) forced to swim at 1.0 body length per second ($BL.s^{-1}$) were double those of controls maintained in still water, and, in salmonids of length 120 - 180mm, velocities of 1.0 to 1.5 $BL.s^{-1}$ have generally been found to result in the highest growth rates (Davison & Goldspink 1977; Jorgensen & Jobling 1993; Hammer 1995). Rates of growth are reduced in fish forced to swim at velocities above the optimum, at least partly due to reductions in the efficiency of food conversion (Davison & Goldspink 1977; Davison 1989).

The increase in mass due to exercise training can consist almost entirely of additional muscle tissue. Totland *et al.* (1987) found that the improvement in muscle growth in Atlantic salmon (*Salmo salar*) resulting from sustained swimming was

achieved mainly by an increase in the rate of hypertrophy of existing fibres, rather than recruitment and growth of new fibres. Kiessling *et al.* (1991) also found that high growth rate in Atlantic salmon was associated with a relatively increased hypertrophic component of muscle growth, although in their study different rates of growth were induced by varying the ration level. In contrast, other studies have found that faster-growing individuals recruit a greater number of fibres to produce a given quantity of muscle (Weatherley *et al.* 1979; Higgins & Thorpe 1990), perhaps because new, small fibres can grow more quickly than older, larger fibres (Weatherley *et al.* 1988).

Steady swimming at low (1.5 - 2.5 $BL.s^{-1}$) cruising velocities is generally believed to be achieved entirely by contraction of the red layer of muscle (Johnston *et al.* 1977; Johnston & Moon 1980a; 1980b; Jayne & Lauder 1994), and the amount of this tissue as a proportion of the total muscle mass can increase with training (Broughton *et al.* 1980; Sänger 1992; Young & Cech 1993). However, Davie *et al.* (1986) suggested that, in rainbow trout, white muscle may be activated at swimming velocities as low as 1.0 $BL.s^{-1}$. In addition, the frequency of bouts of irregular, or burst, swimming can increase with exercise training and result in growth of the white muscle tissue (Totland *et al.* 1987; Hinterleitner *et al.* 1992).

The trout (*Salmo trutta*) spawns in shallow streams, which can experience substantial variations in environmental temperature between years. Patterns of embryonic muscle development in this species have been shown to be affected by rearing temperature (Chapter 5), and early thermal experience may also partly determine the potential for growth in later life. The aim of this experiment was to examine the effect of embryonic thermal experience on later potential for growth in response to different exercise regimes.

## Materials and Methods

### *Fish husbandry and experimental design*

The experiment described in this chapter used fish originating from eight of the ten maternal groups produced from the 1996 fertilisation described in Chapter 2

(see p. 51-52). The mean fork length of the eight relevant female parents was 436 ± 18 mm (S.E.) (see also Appendix I). Only two of the female parents obtained in 1996 proved to be anadromous; although their offspring were included in the present study, the effects of maternal migratory type on the phenotype of the offspring have not been examined. Each of the eight maternal groups was subdivided among egg trays supplied with river water at two temperatures, 10°C (range: 10.0 - 10.4°C) and 2°C (range: 1.6 - 2.4°C), until ready to begin feeding. This stage of development, judged on the basis of depletion of the yolksac, occurred at 78 and 235 days post fertilisation at 10°C and 2°C respectively. At first feeding, groups of fish with different embryonic thermal histories (referred to henceforth as 'temperature groups') were transferred to identical 1m diameter tanks, stocked at similar densities and reared under controlled environmental conditions. The temperature in these tanks was raised from that of the embryonic rearing temperature to 13°C, at the rate of 0.5°C.day$^{-1}$. Water was supplied at 5 l.min$^{-1}$; photoperiod was 16L:8D. Salmon starter food (Ewos, Bathgate) was supplied at approximately 5% body mass.day$^{-1}$.

Exercise experiments involving fish from each of the two temperature groups were begun when the fish had reached a fork length range of 65 - 200mm (mean length = 120 ± 1.6mm S.E. / 124 ± 1.6mm S.E. for the 10°C and 2°C groups respectively). This occurred 305 days after first feeding, in November 1997, for the fish which had been reared at 10°C, and 288 days after first feeding, in March 1998, for the fish which had been reared at 2°C. For each temperature group, a subset of 70 fish in the length range 130 - 165mm was selected from the stock tanks. Of these, 24 fish were sampled immediately (see below). The remaining fish were weighed and measured for fork length, marked with a unique combination of Alcian blue dye spots on the ventral body surface using a panjet dental innoculator (Wright Dental Co., Dundee), and divided between two 1m diameter tanks, similar to those in which they had been previously incubated. In one of the experimental tanks, the 'low-flow' tank, the inflow pipe was adjusted so that water was deflected against the inside face of the tank, diminishing the normal pattern of annular water flow in the tank. In the second, 'high-flow' experimental tank, the annular flow rate was augmented using a submersible pump (ABS Robusta 100 TS) operated at a flow rate of approximately 50 l.min$^{-1}$. Rates of annular water flow, measured using a miniflow probe (George

Kent (Stroud) Ltd.), ranged from 70 $mm.s^{-1}$ at the inner periphery to 180 $mm.s^{-1}$ at the outer periphery in the low-flow tank, and from 120 $mm.s^{-1}$ to 270 $mm.s^{-1}$ in the high-flow tank. Approximately midway through the experiment (on Day 20 for the 10°C group and Day 25 for the 2°C group), fish swimming movements in low and high flow tanks were video recorded (50 $frames.s^{-1}$) using a tripod-mounted Panasonic F10 recorder. Fish were maintained in the experimental tanks for 40 days and then sampled.

***Sampling and tissue preparation***

Fish were killed by overdose of MS-222 (ethyl m-aminobenzoate), identified from dye spot codes, weighed and measured for fork length. The percentage increases in fork length and mass of individual fish were calculated from measurements made at the beginning and end of the exercise period. The condition factor for each fish was calculated as:

$$\text{Condition factor} = \frac{100 \times \text{Mass (g)}}{(\text{Fork length (cm)})^3}$$

Two transverse steaks approximately 2-3mm in thickness were cut at the level of, and posterior to, the anus. An acetate transparency was placed over the anterior face of the more anterior steak and the outline of the entire white muscle was traced using a fine permanent marker. The muscle tissue of one epaxial (dorsal) quadrant of this steak was dissected, mounted on a cork square using Cryomatrix embedding resin (Shandon) and rapidly frozen in liquid isopentane cooled to near its freezing point in liquid nitrogen. The muscle blocks were stored in liquid nitrogen prior to being sectioned transversely at 10μm thickness in a cryostat. Sections were stained with Harris haematoxylin (Sigma) (Fig. 6.1).

The more posterior steak was fixed whole in several washes of buffered 10% formalin (pH 7.2) over two days and stored in 0.1% sodium azide in phosphate buffered saline (pH 7.2).

***Analysis of muscle structure***

For each fish, the area of the tracing of the white muscle outline (i.e. the white muscle cross-sectional area, or CSA) was measured using a drawing pad attached to a Video-Plan Image Analysis System (Kontron Electronics, Basel). Sections were examined under a microscope at x10 magnification, and images each representing an area of 0.474 $mm^2$ were captured using a JVC TK-F7300U frame capture camera. The images were analysed using a Tema v.1.00 image analysis system (Scanbeam, Hadsund, Denmark). For each section, nuclei were counted in 7 - 18 images captured from throughout the epaxial quadrant; the number of images used depended on the size of the section. The mean number of nuclei present per image was calculated, and a value of total number of nuclei per myotome was estimated.

The outlines of white muscle fibres were traced on-screen from similar images and the cross-sectional areas of the fibres were calculated. Between 7 and 18 images were captured from each fish for fibre size analysis, and measurements of fibre areas were made from successive images chosen at random, until the cumulative mean fibre area was deemed to have stabilised i.e. the cumulative mean fibre area did not change in a consistent direction, or by more than approximately 75 $\mu m^2$, with analysis of additional images. The total number of fibres measured per fish ranged from 881 - 2097 (mean = 1161 ± 327 S.E.). The cumulative mean fibre area was divided into the value of white CSA to produce an estimate of the number of white muscle fibres (N) present in the myotome.

The mean area of the largest 50,000 white fibres in the myotome (A) was estimated by ranking the individual fibre area measurements obtained, and then calculating the mean area of the largest *n* fibres measured, where:

$$n = \frac{50{,}000}{N} \times \text{Number of fibres measured}$$

This enabled examination of rates of fibre hypertrophy independently of the rate of fibre recruitment, as described by Johnston *et al.* (1998). All fish examined had fibres in excess of this number (minimum value of N = 61,739 fibres), and so the mean area of the largest 50,000 fibres can be used to describe levels of hypertrophy in the majority of the fibres present before the start of the exercise experiment. The

percentage of 'small' fibres (i.e. fibres of cross-sectional area < 100μm$^2$) in the myotome was calculated and used as a measure of rates of recruitment of new fibres.

Due to difficulties in obtaining sectioned red muscle tissue of sufficient quality, the cross-sectional areas of individual red fibres were not measured. The total cross-sectional areas of the red and of the white muscle were measured from one epaxial quadrant of each of the formalin-fixed blocks of tissue, using a dissecting microscope attached to the frame capture camera and Tema image analysis system. The values of total red muscle cross-sectional area (red CSA) thus produced were adjusted using a fixed-to-fresh correction factor, calculated using the values of white CSA produced from the formalin-fixed blocks and from the acetate tracings of the fresh blocks of tissue.

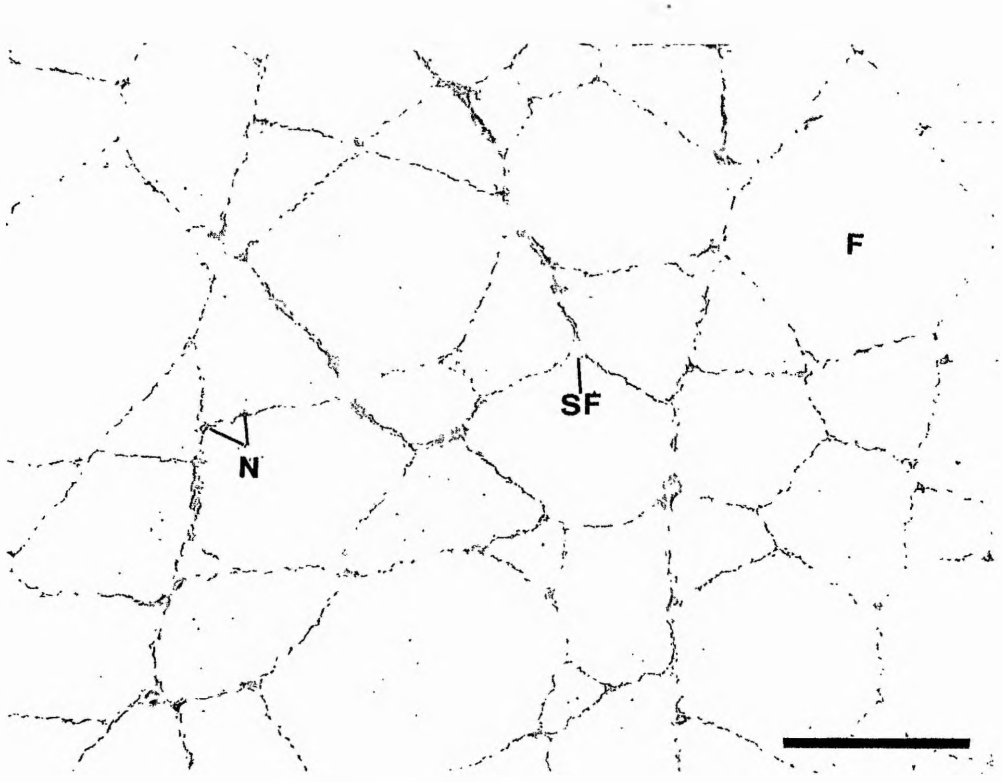



**Fig. 6.1.** Transverse frozen section through white muscle of trout, stained with haematoxylin. F: white muscle fibre; N: nuclei; SF: small fibre (i.e. fibre of cross-sectional area <100μm$^2$). Scale bar = 100μm.

***Measurements of tailbeat frequencies***

For each of the experimental groups, mean tailbeat frequencies (TBFs) were measured from 10 fish judged to be representative of the group. The number of video frames required for a fish to complete a given number of tailbeats was counted and used to calculate the TBF. The minimum number of consecutive tailbeats examined ranged from 20 to 60, depending on the consistency of the TBFs calculated after each consecutive ten tailbeats examined. The specific swimming velocity of the fish, V (in $BL.s^{-1}$) was calculated after Bainbridge (1958), using the formula:

$$V = \frac{3(TBF) - 4}{4}$$

***Statistical analysis***

Tailbeat frequency data were analysed by two-way ANOVA, using embryonic temperature and water flow regime as main effects factors, with the aid of the Minitab statistical analysis package (Minitab Inc., USA). Percentage increases in fork length and in body mass over the course of the exercise experiment were arcsine-transformed, using the following formula after Zar (1996):

$$(\text{Transformed } x) = \arcsin(\sqrt{x})$$

The transformed values were then compared by two-way GLM (general linear model) ANOVAs, using temperature group and flow regime as factors, and the length or mass of individual fish at the beginning of the experiment as a covariate. In order to compare the starting fork lengths and mass values of fish in the four experimental groups (i.e. high- and low-flow groups for each of two temperature groups) with each other and with those of the two initial samples, one-way ANOVAs were used, with the main effect factor containing values ranging from 1 to 6 representing each of the six groups. The cross-sectional areas (CSAs) of white and of red muscle of sampled fish, and the percentages of small fibres, were examined by similar one-way ANOVAs (the percentages of small fibres were arcsine-transformed prior to this analysis). These were followed by Tukey multiple comparison tests

between data for the initial samples in each of the two temperature groups, and between the initial, high-flow and low-flow samples within each temperature group.

The relationships of body mass, white CSA and red CSA with fork length, and of red CSA, N and A with white CSA, were examined by linear regression. Comparisons of the regressions between the six groups were carried out using ANCOVA after Zar (1996). The data were first examined for significant differences in the slopes of the regressions. Where such differences were found, Tukey multiple comparison tests were employed to determine whether one regression differed significantly in slope from the others; where this proved to be the case, that regression was removed and the ANCOVA repeated on the remaining regressions. Once no further significant differences in slopes were found, the second part of the ANCOVA was used to test for significant differences in the elevations of the regressions, followed by Tukey multiple comparison tests for pairwise comparisons of elevations.

The relationships between the percentage of small fibres and white muscle CSA, and between the percentage of small fibres and the percentage increase in body mass during the experiment, were examined by Pearson's correlation.

## Results

### *Tail beat frequencies*

Mean tail beat frequencies (TBFs) and estimated specific swimming velocities for the four experimental groups are presented in Fig. 6.2. Mean TBFs of fish maintained under the high-flow and low-flow regimes differed significantly ($P<0.001$), but did not differ between temperature groups, nor was there was any significant interaction between flow regime and temperature group. Estimated specific velocities ranged from 0.12 - 0.73 $BL.s^{-1}$ in the low-flow tank, and from 0.73 - 1.64 $BL.s^{-1}$ in the high-flow tank. Assuming a fork length of 155mm (the mean fork length over the course of the experiment), this indicates that swimming velocities ranged from approximately 20 - 110 $mm.s^{-1}$ in the low-flow tank, and from 110 - 250 $mm.s^{-1}$ in the high-flow tank.

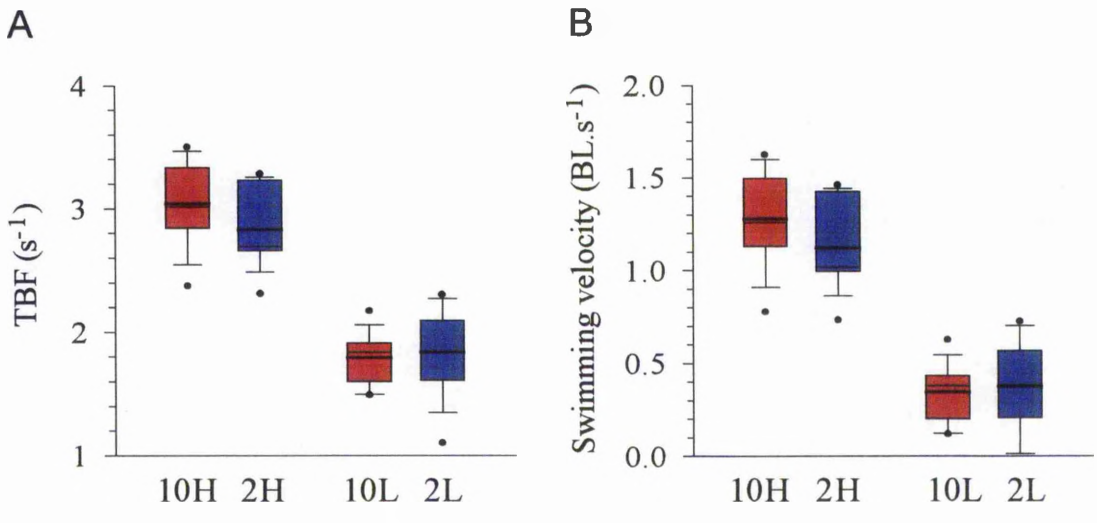



**Fig. 6.2.** Boxplots of (**A**) tailbeat frequency (TBF) and (**B**) specific swimming velocity estimated after Bainbridge (1958). The boundaries of the boxes represent the 25$^{th}$ and 75$^{th}$ percentiles; error bars and closed circles represent 10$^{th}$ and 90$^{th}$ percentiles and outlying points respectively. Thick and regular horizontal bars indicate the mean and median value respectively. 10H: 10°C high-flow regime. 2H: 2°C low-flow regime. 10L: 10°C low-flow regime. 2L: 2°C low-flow regime. Temperatures refer to embryonic thermal history. n = 10 for each group.

***Somatic growth***

At the beginning of the exercise experiment, values of fork length and mass did not differ significantly between the fish sampled initially and those allocated to high- and low-flow tanks. The mean fork length across the groups at the beginning of the experiment was 146 ± 0.9mm (S.E.); the mean mass was 38.3 ± 0.8g.

Analysis of the percentage increases in fork length and mass indicated significant effects of embryonic temperature ($P<0.001$ in both cases) and flow regime (length: $P=0.021$; mass: $P<0.001$), and an interaction between temperature and flow on mass (mass: $P=0.012$) (Table 6.1). Fish which had been reared at 10°C as embryos grew more than those which had been reared at 2°C, and higher mean growth rates were observed among fish in the high-flow tanks (Fig. 6.3). Percentage increases in length and mass did not vary significantly with the size of the fish at the beginning of the experiment.

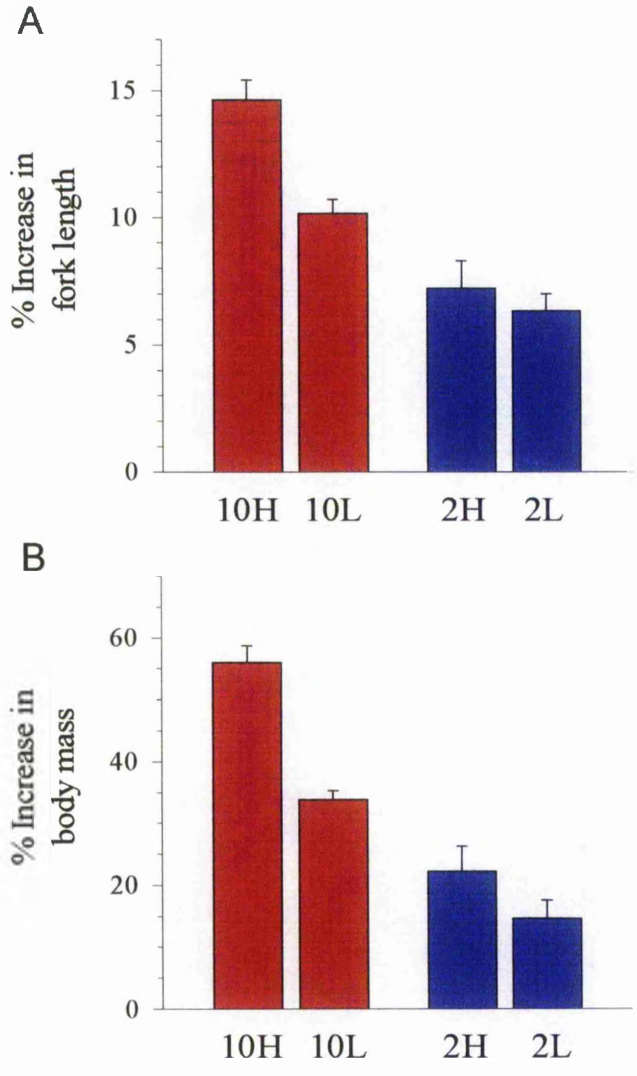



**Fig. 6.3.** Mean values of (**A**) percentage increase in fork length and (**B**) percentage increase in body mass. 10H: 10°C high-flow regime. 10L: 10°C low-flow regime. 2H: 2°C low-flow regime. 2L: 2°C low-flow regime. Temperatures refer to embryonic thermal history. Error bars are standard errors, derived from arcsin-transformed data and then backtransformed.

The slope of the regression of body mass against fork length was significantly higher for the 10°C high-flow group than for the other five groups ($P<0.001$ for all multiple comparisons) (Fig. 6.4). Comparison of the elevations of the remaining regressions revealed small but significant ($P<0.001$) differences. Comparing the initial samples, the fish which had been reared at 2°C as embryos were significantly heavier, relative to fork length, than fish which had been reared at 10°C ($P=0.025$). The mean condition factors for the 2°C and 10°C initial samples were 1.23 ± 0.01 S.E. and 1.17 ± 0.01 S.E. respectively. In addition, after 40 days in the low-flow regime, fish from the 10°C temperature group had gained mass relative to length ($P=0.05$), but fish from the 2°C group had lost mass relative to length ($P=0.025$).

***Muscle cross-sectional area***

When the experiment began, mean values of muscle cross-sectional area (CSA) were not significantly different between temperature groups, for either white or red muscle. Over the course of the experiment, only the fish in the 10°C high-flow group showed significant increases in mean white CSA (P-ANOVA<0.001) and mean red CSA (P-ANOVA<0.001) when compared to the relevant initial sample (Fig. 6.5a, b). (Note: P-values are not available for individual Tukey comparisons on one-way ANOVAs).

When muscle cross-sectional area was examined relative to length, however, it was found that, at the start of the experiment, the fish which had been reared at 2°C as embryos had significantly more red muscle than those which had been reared at 10°C ($P=0.01$). This difference between temperature groups in red CSA amounted to approximately 0.03 $cm^2$, almost 50% of the total red CSA in the smallest 10°C fish. By the end of the experiment, however, there were no longer any significant differences in the area of red muscle between temperature groups. In addition, relative to length, fish in the 2°C low-flow group had less white muscle than those sampled when the experiment began ($P=0.05$).

The relationship between white CSA and red CSA did not vary significantly among any of the groups examined.

**Table 6.1**: Results of two-way GLM ANOVAs examining differences in percentage increases in fork length and body mass with embryonic temperature and water flow regime.

| | | % Increase in fork length | | % Increase in mass | |
|---|---|---|---|---|---|
| Source | D.F.[a] | *F*-ratio | P | *F*-ratio | P |
| Temperature group (T)[b] | 1 | 45.15 | <0.001 | 68.21 | <0.001 |
| Flow regime (F) | 1 | 5.57 | 0.021 | 23.66 | <0.001 |
| T * F | 1 | 3.64 | ns | 6.72 | 0.012 |
| Initial length / mass | 1 | 1.33 | ns | 0.89 | ns |
| Error | 77 | | | | |
| Total | 81 | | | | |

[a] D.F. = Degrees of freedom. [b] Refers to the embryonic temperature regime.

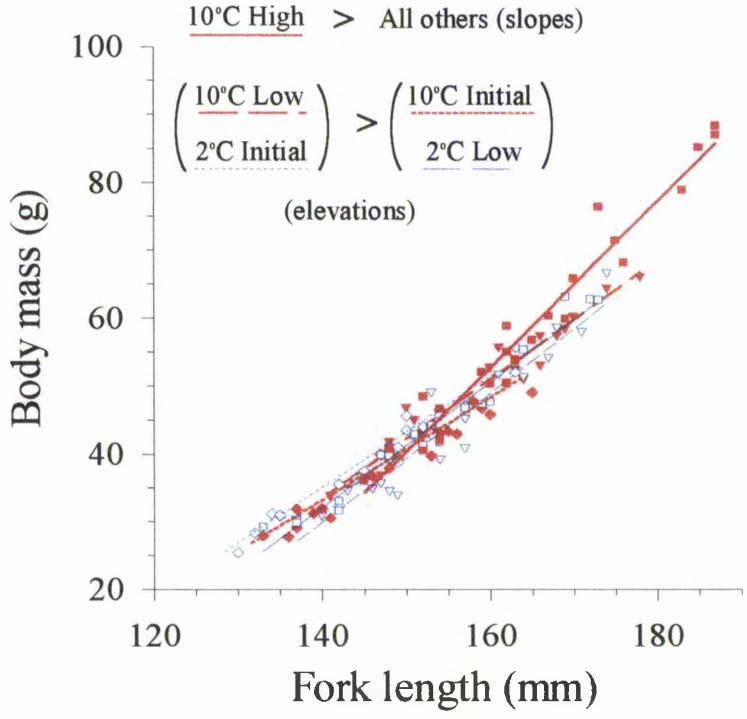



**Fig. 6.4.** Scattergram, with linear regressions, of the relationship between body mass and fork length. The text within the graph refers to significant differences in slopes and elevations of regressions. 10°C groups: Red, closed symbols, thick lines. 2°C groups: Blue, open symbols, regular lines. Initial samples: Diamonds, dotted lines. High-flow groups: Squares, solid lines. Low-flow groups: Inverted triangles, dashed lines.

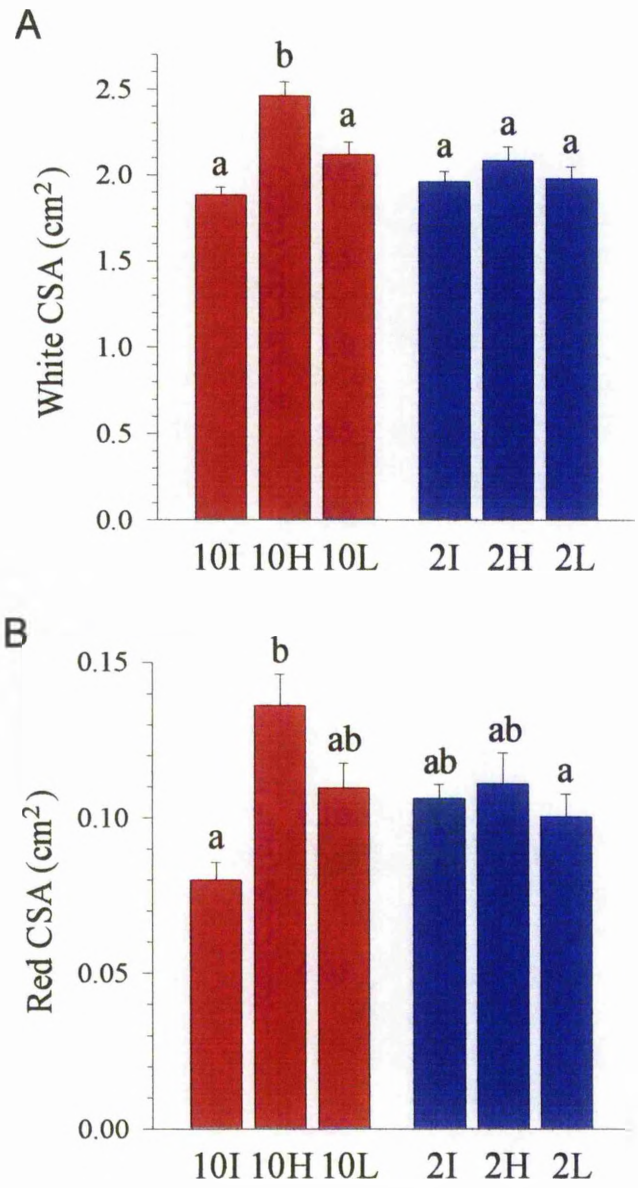



**Fig. 6.5.** Mean values of (**A**) white muscle cross-sectional area (CSA) and (**B**) red muscle cross-sectional area. Significant differences between columns are denoted by the lack of a common lower-case letter. 10I: 10°C initial sample. 10H: 10°C high-flow regime. 10L: 10°C low-flow regime. 2I: 2°C initial sample. 2H: 2°C high-flow regime. 2L: 2°C low-flow regime. Temperatures refer to embryonic thermal history. Error bars are standard errors.

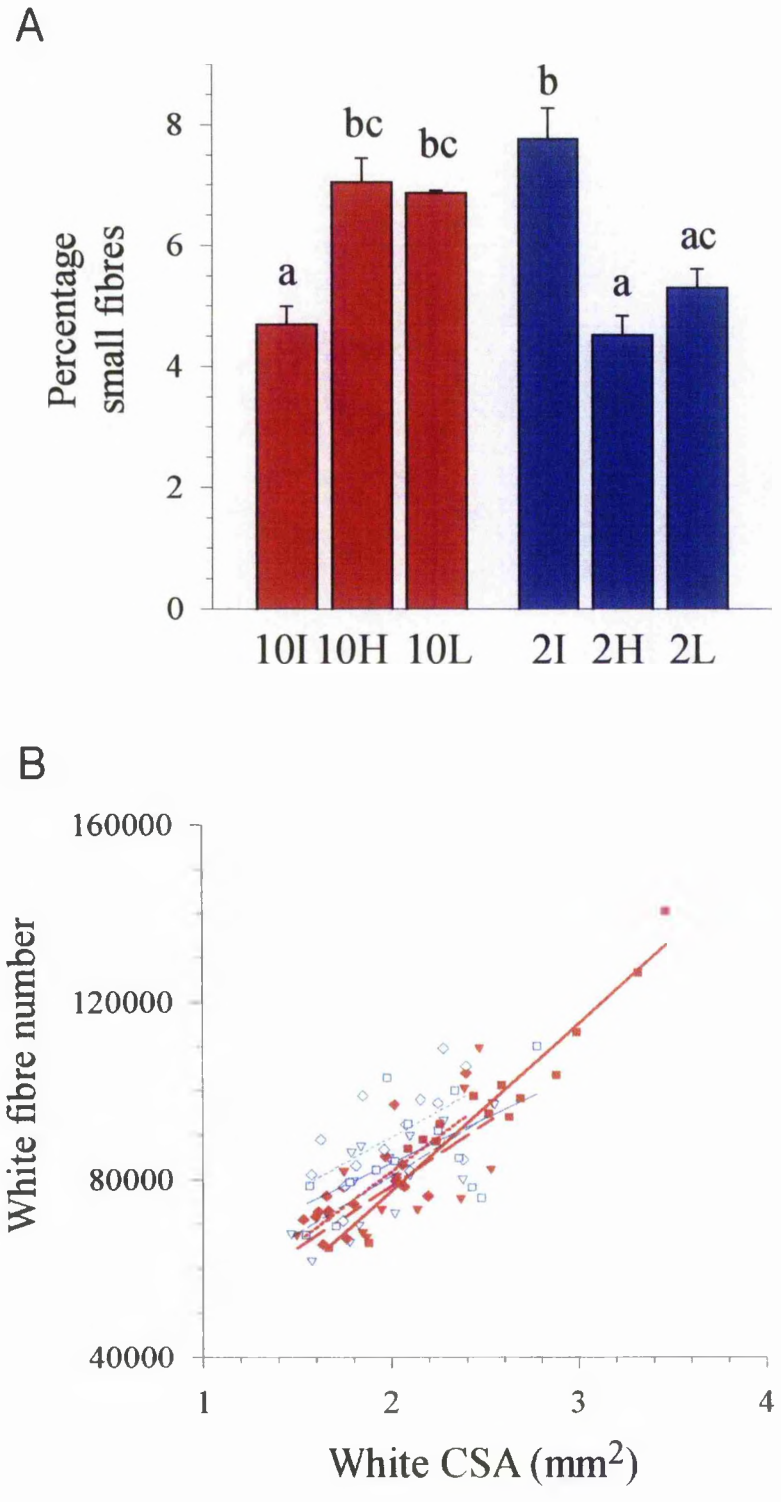
***Muscle cellularity***

Fish in the 2°C initial sample had almost twice as many small muscle fibres (i.e. fibres of cross-sectional area < 100μm$^2$) as those in the 10°C initial sample (P-ANOVA<0.001)(Fig. 6.6a). This implies that rates of fibre recruitment, at least in the period immediately prior to the experiment, varied with embryonic thermal regime. Recruitment of new fibres during the exercise experiment also differed substantially between the two temperature groups - the percentage of small fibres showed a significant increase in both high- and low-flow groups at 10°C, but decreased significantly in both groups at 2°C (Fig. 6.6a). Although not significantly related to the total amount of white muscle, the percentage of small fibres was correlated with the rate of increase in body mass (Pearson's correlation, $r$ = 0.283, P=0.05), indicating a link between somatic growth rate and the rate of recruitment of new muscle fibres.

However, the mean percentage of fibres in the 'small' category, for all fish examined, was just 6.0% ± 0.2% S.E., and the differences between the temperature groups in rates of fibre recruitment were not reflected in the total number of white muscle fibres (Fig. 6.6b). When total fibre number was examined relative to white CSA, the regressions for the 10°C and 2°C initial samples were not significantly different, nor were the regressions for the initial samples different from those taken at the end of the exercise experiment, in either of the temperature groups. Estimated values of total fibre number ranged from approximately 60,000 in fish with 1.5cm$^2$ of white muscle, to approximately 140,000 in fish with 3.5cm$^2$ of white muscle.

The number of nuclei in the white muscle varied substantially with embryonic thermal regime. At the start of the exercise experiment, fish in the 10°C initial sample had significantly higher numbers of nuclei in the white muscle than those in the 2°C initial sample, relative to white CSA (P<0.001) (Fig. 6.7). This contrasts with the situation at first feeding, when nuclear density was lower in the 10°C fish (see Chapter 5). At the end of the experiment, the 10°C high-flow group had maintained this high density of nuclei; however, values of nuclear number relative to white CSA in the 10°C low-flow group were lower than in the 10°C initial sample (P<0.001), resembling those of fish in the 2°C groups.

**Fig. 6.6. A**: Mean percentages of small (cross-sectional area <100μm$^2$) white fibres. 10I: 10°C initial sample. 10H: 10°C high-flow regime. 10L: 10°C low-flow regime. 2I: 2°C initial sample. 2H: 2°C high-flow regime. 2L: 2°C low-flow regime. Temperatures refer to embryonic thermal history. Significant differences between columns are denoted by the lack of a common lower-case letter. Error bars are standard errors, derived from arcsin-transformed data and then backtransformed. **B**: Scattergram, with linear regressions, of the relationship between the number of white muscle fibres and white muscle cross-sectional area (CSA). 10°C groups: Red, closed symbols, thick lines. 2°C groups: Blue, open symbols, regular lines. Initial samples: Diamonds, dotted lines. High-flow groups: Squares, solid lines. Low-flow groups: Inverted triangles, dashed lines.


A
Percentage small fibres
0
2
4
6
8
a
bc
bc
b
a
ac
10I 10H 10L 2I 2H 2L
B
White fibre number
40000
80000
120000
160000
1
2
3
4
White CSA (mm2)

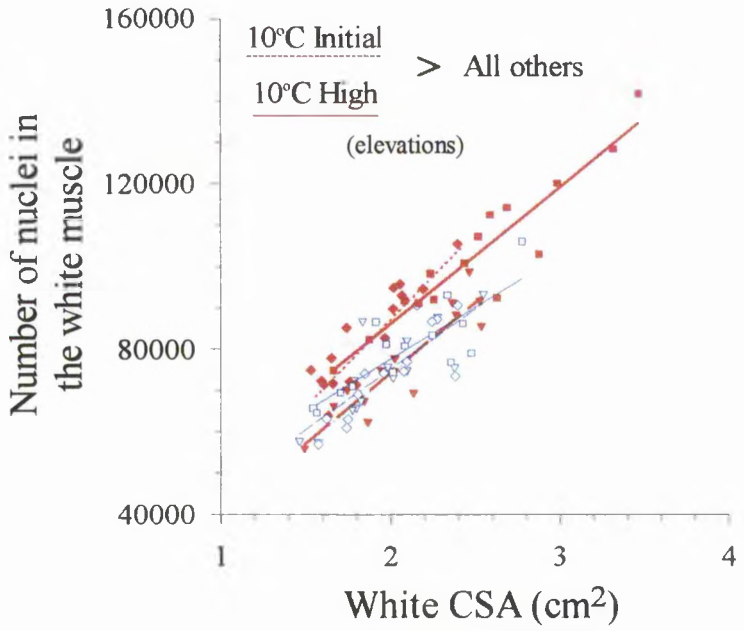



**Fig. 6.7.** Scattergram, with linear regressions, of the relationship between number of nuclei in the white muscle and white muscle cross-sectional area (CSA). The text within the graph refers to significant differences in regression elevations. 10°C groups: Red, closed symbols, thick lines. 2°C groups: Blue, open symbols, regular lines. Initial samples: Diamonds, dotted lines. High-flow groups: Squares, solid lines. Low-flow groups: Inverted triangles, dashed lines.

The slope of the regression of mean area of the largest 50,000 white fibres (A) against CSA was lower in the 10°C high-flow group than in the other five groups ($P<=0.05$ for all comparisons) (data not shown). No significant differences in elevation were found between the remaining five regressions after removal of the 10°C high-flow data. Values of A ranged from approximately 3,000$\mu m^2$ in fish with 1.5$cm^2$ of white muscle, to approximately 5,500$\mu m^2$ in fish with 3.5$cm^2$ of white muscle.

## Discussion

The fish which had been reared at 10°C as embryos exhibited a greater potential for somatic growth during the exercise experiment, when compared to fish which had been reared at 2°C. The greater growth of the fish in the 10°C groups included much higher rates of muscle fibre recruitment. Alami-Durante *et al.* (1997) also reported an association between growth rate and the rate of formation of new muscle fibres, and Higgins & Thorpe (1990) found that the relative amount of muscle growth due to growth of new, as opposed to pre-existing, fibres was greater at times of fast growth.

The differences in levels of fibre recruitment between temperature groups were not sufficient to produce detectable differences in the total number of fibres. If a regression of fibre number against white CSA is calculated using data from all the groups, even where the regression most accurately describes the relationship, at approximately CSA = 2.5mm$^2$, (fibre number = 98,000), calculated 95% confidence intervals are equivalent to ± 3,000 fibres, or 3.1% of the fibre number in fish of that size. The percentage of small fibres differs by no more than 3.3% between individual groups; therefore, it is not surprising that differences in the proportions of small fibres did not translate into significant differences in total fibre number.

Growth rates were higher among fish in the high-flow regime, which were forced to swim at velocities similar to those found to result in optimum growth in other studies on salmonids of similar size (Davison & Goldspink 1977; Jorgensen & Jobling 1993; Hammer 1995). Jorgensen & Jobling (1993) reported increased food intake and food conversion efficiency, and reduced levels of aggressive behaviour and fin damage, in Atlantic salmon swimming at 1.5 BL.s$^{-1}$, relative to rested controls. Houlihan & Laurent (1987) found that, although rates of protein degradation were increased in rainbow trout forced to swim at 1.0 BL.s$^{-1}$, rates of protein synthesis were increased to an even greater extent, resulting in improved growth rates. In the present study, the ranges of swimming velocities estimated for each of the experimental groups, based on tailbeat frequencies and estimated mean fish length, generally corresponded well with the water velocities measured in the

experimental tanks. The lowest estimated swimming velocity in the low-flow regime ($20mm.s^{-1}$) was, however, substantially lower than the minimum recorded water velocity in that regime ($70mm.s^{-1}$); fish in this regime were observed to be capable of resting on the floor of the tank for brief periods of time without swimming.

It has been suggested that the ability of a fish to produce new muscle tissue may be directly linked to the total number of nuclei in the muscle (Matschak *et al.* 1997). The high rates of growth of the fish reared at 10°C as embryos may therefore be associated with the relatively greater density of nuclei in the muscle of these fish, compared to the fish in the 2°C groups, which in turn may reflect the size of the population of myosatellite cells. Johnston (1993) reported that the number of myosatellite cells was three times higher in Atlantic herring reared at 8°C than in those reared at 5°C, and both growth rate and nuclear density in the white muscle of spring-spawning Atlantic herring larvae have been shown to increase with embryonic rearing temperature (Johnston *et al.* 1998). Myonuclear density and muscle size in turkeys have also been found to be associated with the level of proliferation of myosatellite cells during early life stages (Mozdziak *et al.* 1997).

Interestingly, at the end of the exercise experiment, fish in the 10°C high-flow group had maintained their relatively high nuclear density. In contrast, in the fish in the 10°C low-flow group, numbers of nuclei in the white muscle relative to CSA fell to levels similar to those of the 2°C groups. The new fibres recruited by the 10°C low-flow fish must have required at least some new myonuclei. However, the low swimming speeds of these fish, while sufficient to stimulate recruitment of muscle fibres, may have been insufficient to stimulate an equivalent increase in myosatellite replication, and so the total reserve of myosatellites may have been reduced by the end of the experiment. Thus, differences in the potential for growth between fish reared under different conditions as embryos may not be fixed, but may also depend on subsequent environmental conditions.

It is important to note that, at the start of the experiment, the fish which had been reared at 2°C as embryos were heavier relative to length, and had more red muscle and greater rates of fibre recruitment, than those which had been reared at 10°C. It is possible that the greater potential for growth of the fish in the 10°C

groups, as observed during the exercise experiment, may have been suppressed in the period between first feeding and the start of the experiment, perhaps due to the high stocking density of the fish. Although the environmental conditions experienced during this period were intended to be identical for the two temperature groups, small differences in water temperature or feeding intensity may have enabled the fish reared at 2°C as embryos to achieve somewhat greater growth by the time the exercise experiment began.

If there were slight differences in the exact environmental conditions under which the fish were kept between first feeding and the start of the experiment, then it is also possible that the difference in growth potential between the two temperature groups arose during this period, rather than during the embryonic / alevin period. In Chapter 5, it was found that the number of total muscle nuclei, relative to the number of muscle fibres, did not vary with rearing temperature in trout embryos and alevins, suggesting that embryonic rearing temperature did not affect numbers of myosatellite cells. However, in studies on muscle tissue of carp (*Cyprinus carpio*), the proportion of muscle nuclei belonging to myosatellite cells was found to be less than 8% (Koumans *et al.* 1994); small differences in the myosatellite population in first-feeding trout reared at different temperatures may therefore have gone undetected. The results described in Chapter 5 include evidence of higher rates of post-hatch muscle growth, relative to length, in trout reared at 10°C than at 2°C; this 'catch-up' growth may be indicative of the greater underlying growth potential of the 10°C-reared alevins. The large difference in embryonic rearing temperature seems the most likely causative mechanism behind the differences in myonuclear density and growth potential between the two sets of fish examined in this chapter.

# Chapter 7: General discussion

The results presented in Chapters 2 - 6 demonstrate a wide range of effects of temperature on early development and growth in the trout (*Salmo trutta*). One of the key features of the studies described in this thesis is the emphasis on development, or differentiation, as a process distinct from that of growth; the relationship between the two processes has been shown to vary with factors both environmental, such as temperature, and inherited, such as egg size (Chapter 3). There is also potential for variation within the developmental sequence itself, such as the earlier onset of hatching at high temperatures, although development in trout was shown to be relatively canalised when compared to that of other fish species (Chapters 2 & 4). It was found in Chapter 5 that muscle growth of trout during early life stages varies with temperature, both in terms of the total amount of muscle produced and the muscle cellularity, particularly around the time of hatching. In addition, the temperature at which trout embryos were reared was found to modulate their potential for growth in later life (Chapter 6), and it was suggested that this effect may be mediated through temperature effects on the population of myosatellite cells.

The methods used for staging the development of teleost embryos have generally changed little over the last 50 years or more, in contrast to the great advances in the level of detail in studies of fish embryology (e.g. Kimmel *et al.* 1995). Balon (1971; 1975b; 1990) has repeatedly described the drawbacks inherent in the use of a series of disconnected 'stages' to describe the continuous changes that occur during embryonic development; nevertheless, stages such as those described by Battle (1944) over fifty years ago for Atlantic salmon are still being described today (e.g. Iwamatsu 1994). In this study, developmental 'steps', as recommended by Balon (1975b; 1990), have been described for *Salmo trutta*, in addition to a developmental scoring system which attempts to advance the underlying concept behind steps (i.e. that development is an integrated and continuous process), and which represents an entirely novel approach to the problem of categorising early life stages of fish. This scoring system enabled the process of development to be studied in a quantitative way, side by side with the study of growth, as in Chapter 3.

Although the relative timings of certain developmental changes, such as the onset of movement, were found to vary with temperature (Chapter 2), organogenesis in the trout, including development and innervation of the first muscle tissue (Chapter 4), was found to be remarkably entrained, particularly when compared with other teleost species such as the Atlantic herring (Johnston 1993; Johnston *et al.* 1995). Salmonid embryos usually develop in shallow streams which experience substantial variation in temperature, even over short periods of time. Temperature records of water taken from the river Almond during the present study frequently showed fluctuations of 3-4°C within 24-hour periods, and daily fluctuations of as much as 7-8°C have been recorded. In such a variable environment, continual adjustments to the developmental sequence in response to changes in temperature could result in severe disruption of embryogenesis. Other salmonid species have also generally been found to show only limited shifts in the relative timing of developmental changes with temperature (Vernier 1969; Ballard 1973c; Gorodilov 1989). This suggests that members of the Family Salmonidae may have evolved strict mechanisms to regulate the sequence of development at different temperatures.

The relationship between overall development and growth, however, and the use of endogenous reserves to achieve both, was found to vary substantially with temperature (Chapter 3). A shift towards greater developmental advancement but reduced growth was observed in trout alevins reared at relatively high temperatures. Whether this change in the pattern of resource utilisation is adaptive (i.e. a response that results in increased fitness and which has arisen by natural selection), or merely due to differential effects of temperature on the cellular mechanisms involved in growth and development, is not clear. Reductions in size relative to degree of development at high temperatures have been described in a range of species (Ray 1960; von Bertalanffy 1960; Atkinson 1994; Atkinson 1996), although no single hypothesis has yet been proposed which satisfactorily explains this phenomenon.

One particularly interesting finding described in Chapter 3 was that an increase in temperature resulted in greater efficiency of yolk use for development, but that a reduction in egg size resulted in an equivalent decrease in the level of development achieved from the yolk. For a number of fish species, as the ambient temperature increases, mean egg size decreases (Blaxter & Hempel 1963;

Tanasichuk & Ware 1987; Mihelakakis *et al.* 1995; Imai & Tanaka 1997), and egg size in trout has been found to be relatively lower, and fecundity higher, in fish exhibiting greater growth rates (Lobon Cervia *et al.* 1997), such as would be expected at higher temperatures. This variation in egg size may have evolved partly to ensure that trout alevins reach the same level of advancement at the point of yolk exhaustion regardless of environmental temperature, albeit at the expense of growth at high temperatures and of fecundity at low temperatures.

It has been suggested that environmental conditions such as temperature could partially determine the migratory type of trout (Jonsson 1985). Nordeng (1983) found that the proportion of resident fish within a group of Arctic charr increased with feeding intensity, and residency is more common among trout populations found at lower latitudes (Elliott 1994); both observations suggest a link between growth rate and life history strategy. Overall rates of growth increase with environmental temperature; however, in the present study, the greater long-term growth potential of fish reared at a high temperature as embryos only became manifest under certain conditions (i.e. those experienced during the course of the exercise experiment) (Chapter 6). Life history strategy of trout may, therefore, depend on a combination of both temperature and other environmental conditions such as water flow rate, food availability and levels of intra-specific competition. It was unfortunate that the effect of maternal migratory type could not be effectively studied in the fish from the 1996 fertilisation (Chapters 3 and 6); however, in Chapters 2, 4 and 5, only minimal differences in early growth and development were found between offspring of anadromous and of freshwater resident females, for example the significantly greater length of offspring of resident females at hatch. Maternal influences on migratory type may not become manifest until after first feeding, and examination of greater numbers of families may also uncover differences in development with maternal migratory type which may have escaped detection in the present study. Alternatively, it has been suggested that the migratory type of the female parent may influence that of the offspring through differences in selection of spawning sites, which may lead to associated differences in water flow rates (Thompson 1995). The inheritance of anadromy may also be mediated through egg size, which was found in the current study to be a major factor in determining growth prior to first feeding. However, if mean egg size was relatively higher in

anadromous females, as was found by Elliott (1994), and growth during the first year was thus improved, the evidence to date would seem to suggest this would increase the tendency to freshwater residency, rather than anadromy.

It was also found that rearing temperature can affect patterns of growth within individual tissues such as muscle. The findings of Chapter 5, which indicate that muscle growth was reduced at 10°C relative to that at 2°C by the end of the embryonic period, might appear to contradict the finding in Chapter 3 that, prior to the 'hatch' stage, conversion of yolk into body mass was more efficient at the higher temperature. The work described in these two chapters was carried out on fish reared in different years, which may partially explain this apparent discrepancy. However, it must be remembered that, in Chapter 5, muscle cellularity was examined relative to development, and to length, both of which vary in their relationship with yolk utilisation in a temperature-dependent manner. Perhaps, in all future studies on any such aspect of growth prior to first feeding, yolk usage should also be considered, and used as the primary determinant against which variables both of growth and development should be compared. In addition, body mass need not necessarily be an accurate indicator of the quantity of muscle present. The priority placed on muscle growth, as a proportion of total growth, may well vary both with temperature and with development. Muscle tissue may be of relatively low importance around the time of hatching, when the alevins remain concealed among the gravel, but may become crucial at the time of emergence; hence, the 'catch-up' in muscle growth observed at 10°C during the alevin period. This 'catch-up' involved extra recruitment of both embryonic and post-embryonic fibres, suggesting that, although the two populations are recruited at different times, and also differ in their placement within the myotome, they may both respond to the same cues for growth.

The shift towards reduced fibre recruitment relative to fibre hypertrophy observed in trout reared at high temperature (Chapter 5) has also been found in other salmonid species (Stickland *et al.* 1988; Matschak *et al.* 1998). This has been described as a response to reduced availability of resources for growth at high temperatures, with hypertrophy being considered less 'metabolically expensive' than formation of new fibres (Usher *et al.* 1994). While efficiency of yolk use does not appear to be a limiting factor at 10°C, relative to 2°C, during the embryonic period

(Chapter 3), availability of oxygen may partly determine muscle cellularity (Matschak *et al.* 1995; Matschak *et al.* 1997; Matschak *et al.* 1998). Alternatively, more fundamental mechanisms at the cellular level may be involved. Van der Have & de Jong (1996) theorised that changes in temperature may affect cell growth to a lesser extent than cell division because the limiting step in protein deposition involves diffusion of ribosome subunits, and rates of diffusion are relatively insensitive to temperature changes. Nuclear division, on the other hand, is limited by the enzymatic speed of DNA polymerases, which have high $Q_{10}$ values and are therefore more strongly influenced by environmental temperature.

While relatively high environmental temperatures may result in somewhat reduced overall growth by the time the yolk is exhausted (Chapter 3), the potential for growth in later life appears to be improved by such conditions (Chapter 6). Although the mechanism behind this phenomenon has not yet been determined, the high nuclear density in the muscle of the fish reared at 10°C as embryos suggests that differences in the population of myosatellite cells, such as those described by Johnston and co-workers (Johnston 1993; Johnston *et al.* 1998), are involved. Myosatellite cells were not quantified in the present study, as the electron microscopy required would have been extremely time consuming, given the large size of trout embryos when compared to those of, for example, Atlantic herring. Although a possible technique for immunocytochemical identification of myosatellite cells (after Zhang & McLennan 1994) was explored without success during the course of the present study, a new immunocytochemical method has recently been developed which promises to enable quantification of myosatellite cells with relative ease (I.A. Johnston, pers. comm.). This method involves labelling of muscle tissue with an antibody specific to the c-met receptor tyrosine kinase, which is present on all myosatellite cells, both quiescent and active, but which is not found on other mononucleate cells within muscle (Cornelison & Wold 1997). This receptor has been implicated in the pathway by which quiescent, 'reserve' myosatellite cells are stimulated to proliferate (Allen *et al.* 1995).

***Possible avenues of future research***

As with all scientific endeavour, the research undertaken for this thesis has the potential for future expansion and improvement. The temperatures at which the fish were reared (10°C, 6°C and 2°C) in Chapters 2 - 6 represent just three points in a continuum of potential temperatures which trout embryos can experience in the wild, ranging from 0°C to 15°C (Elliott 1994). In Chapter 3, it was found that the optimum regime for conversion of yolk into body mass is one where the temperature experienced by the alevin is greater than that experienced by the embryo, suggesting that the effects of variable temperature regimes on development and growth merit further investigation. Patterns of development and growth, and their response to environmental variables, may also differ between stocks, both between and within watercourses. For example, research is currently underway into the effects of early thermal experience on growth potential in populations of Atlantic salmon native to two different tributaries of the River Dee. Early results indicate that the temperature experienced during the embryonic period modulates muscle growth after first feeding in one population, but not in the other (I.A. Johnston, pers. comm.).

In Chapter 3, it was found that temperature, by affecting the efficiency of yolk utilisation, can alter the relationship between the degree of development and the size of the fish. While the advantages of greater size at emergence have been well documented (Chapman 1962; LeCren 1973; Wankowski & Thorpe 1979; Huntingford *et al.* 1990), less is known about the importance of developmental state. Further study is merited to examine which parameters truly determine the fitness of the alevin at, and in the period after, emergence into the stream, and how these may vary with environmental conditions. To answer these questions, the impact of the environment on other aspects of the trout's natural habitat cannot be ignored. For example, how does temperature affect the size of the trout's prey? If an increase in ambient temperatures, perhaps due to global warming, resulted in disproportionate decreases in the size of predator and prey, how would this affect trophic relationships? Integrated ecological studies, on not one but many species, examining the combined effects of changes in several abiotic variables such as temperature, oxygen, and perhaps environmental stability, may prove highly rewarding. Such integrated studies may be particularly useful for examining determination of migratory type, which is likely to depend on a range of variables, both environmental

and genetic. Ultimately, hatchery-based studies such as the present one must be linked to field studies of behaviour in the wild, and its response to different rearing conditions during early life. This will, however, require sophisticated tracking of individual fish over their entire life cycle.

Findings to date on the effects of the environment on muscle cellularity, such as those presented in Chapter 5, are interesting both in themselves, for the information they provide about mechanisms of development and growth, and also commercially. The firmness of cooked fish flesh has been found to be related to the mean size of the muscle fibres (Hatae *et al.* 1990). Somewhat surprisingly, the relationship between structure, in terms of cellularity, and function of fish muscle remains relatively unexplored, although such a study on larvae of Atlantic herring is currently underway. It is perhaps quite revealing about the nature of scientific investigation that, similarly, the effect of temperature on the meristic character of vertebral number has been known for over fifty years (Gabriel 1944), but its impact on swimming ability and survival was not investigated until much more recently (Swain & Lindsey 1984). If muscle cellularity were found to affect swimming performance, it would be interesting to examine whether this was true regardless of whether a particular muscle phenotype arose due to a particular temperature, or a particular diet, or as a result of genetic determination.

The mechanisms controlling activation, proliferation and differentiation of myosatellite cells have not yet been fully elucidated. The patterns of expression of muscle regulatory factors (MRFs) such as myoD and myogenin are proving to be quite complex (Rescan *et al.* 1995; Cossu *et al.* 1996; Cornelison & Wold 1997; Kitzmann *et al.* 1998), and new MRFs are continually being identified (Bassel-Duby *et al.* 1994; Allen *et al.* 1995). Only when these regulatory pathways are more fully understood can we truly hope to understand the mechanisms by which they respond to variables such as temperature. Soon it may be possible to distinguish quiescent myosatellite cells from those which are proliferating or differentiating, and to distinguish myosatellites which will contribute to recruitment of new fibres from those which will contribute to hypertrophy of existing fibres. The dynamics of changes in the muscle cell population in response to environmental variables such as temperature may then be described, from early embryos through to adults. In

addition, *in vitro* experiments may be used to determine how myosatellite cells are affected by their cellular environment, for example by culturing myosatellite cells in the presence of extracts of muscle fibres of different sizes or nuclear densities. Transplantation of myosatellites from muscle of one fibre type to another has already been carried out in experiments investigating the role of innervation on the phenotype of regenerated muscle fibres (Dolenc *et al.* 1994); similar experiments transferring myosatellite cells between fish of differing thermal histories, if possible, may also yield interesting findings on the mechanisms by which early thermal experience alters the potential for future growth.

The recent explosion in the number and variety of techniques available for genetic manipulation have been put to good use in studies on genetic factors affecting the relationship between cell size and cell number in model species such as *Drosophila melanogaster* (Raff 1996; Neufeld *et al.* 1998; Su & O' Farrell 1998), and it is likely that at least some of the regulatory pathways identified are conserved among a broad range of species. Studies examining the genetics of development in fish species, while not as common, have nonetheless provided and will continue to provide valuable information on the development of muscle tissue (Felsenfeld *et al.* 1990; Halpern *et al.* 1993) and muscle innervation (Grunwald *et al.* 1988; Sepich *et al.* 1998), as well as on formation of other elements such as blood and endothelial cells (Stainier *et al.* 1995; Orkin & Zon 1997). Techniques have been developed which allow the programmed activation of particular genes at a point in development controlled by the experimenter (e.g. Weigmann *et al.* 1997); if such methods become available for fish species, they would constitute a powerful tool. For example, if the genes which regulate the switch from embryonic to post-embryonic muscle growth were identified, this transition could be triggered at varying times, and the resulting changes in muscle cellularity analysed.

Clearly, there is almost limitless scope for further study. The challenge for the future will be not only to advance our understanding of the individual topics covered in the present study, but to maintain an integrated approach which combines developmental biology and physiology with ecology, and the theoretical with the applied.

## Appendix I: Sizes of parental fish

**Table I.1.** Fork lengths of parental fish used in the 1995 fertilisation (see also Chapters 2, 4 and 5). A: Anadromous female. R: Freshwater resident female.

| Females | | Males |
|---|---|---|
| Fork length (mm) | Migratory type | Fork length (mm) |
| 580 | A | 520 |
| 580 | A | 170 |
| 460 | A | 180 |
| 310 | R | 165 |
| 385 | R | 245 |
| 505 | R | 145 |
| 390 | R | |
| 420 | A | |
| 250 | R | |
| 410 | R | |
| Mean ± S.E. | | Mean ± S.E. |
| 429 ± 34 | | 238 ± 59 |

Of the ten female parents, four were anadromous (mean fork length = 510 ± 71mm) and six were freshwater residents (mean fork length = 375 ± 80mm). The difference in mean lengths, as examined using a 2-way t-test, was significant (P=0.048).

**Table I.2.** Fork lengths of parental fish used in the first 1996 fertilisation (see also Chapters 2, 3 and 6). A: Anadromous female. R: Freshwater resident female.

| Females | | | Males |
|---|---|---|---|
| Fork length (mm) | Migratory type | Examined in Chapters: | Fork length (mm) |
| 420 | A | 2, 3, 6 | 470 |
| 525 | R | 2, 3, 6 | 390 |
| 435 | R[a] | 2 | 435 |
| 430 | R | 2, 3, 6 | 225 |
| 460 | A | 2, 3, 6 | 180 |
| 400 | R | 2, 3, 6 | 240 |
| 420 | R | 2, 6 | |
| 420 | R* | 2, 3 | |
| 350 | R | 2, 6 | |
| 485 | R | 2, 6 | |
| Mean ± S.E. | | | Mean ± S.E. |
| 435 ± 15 | | | 323 ± 50 |

[a] While analysis of the carotenoid pigment profiles of eggs from these females strongly suggested that they were freshwater resident fish, the migratory type could not be determined with complete certainty. Offspring of these females were not examined in the exercise experiment described in Chapter 6, although it was ultimately decided not to investigate the effects of maternal migratory type on growth potential in that study.

**Table I.3.** Fork lengths of parental fish used in the second 1996 fertilisation (see also Chapter 2). A: Anadromous female. R: Freshwater resident female.

| Female | | Males |
|---|---|---|
| Fork length (mm) | Migratory type | Fork length (mm) |
| 485 | A | 370 |
| | | 240 |

## Appendix II: Determination of maternal migratory type

Prior to collection of eggs, the migratory type of prospective female parents was estimated based on examination of the growth patterns of the scales, as described by Frost & Brown (1967). Maternal migratory type was established definitively by isocratic normal phase HPLC analysis of unfertilised eggs (5-10 per female).

Pigment profiles of eggs collected in 1995 were analysed with the assistance of Dr. Patrick Noack (Dept. of Zoology, University of Aberdeen). The procedure employed is detailed in Noack *et al.* (1996), an extract of which follows:

"{The eggs}... from each female were homogenised in 1 ml acetone (Rathburn, UK) using a 7 mm probe (Polytron, Switzerland). The probe was washed with 1 ml acetone and the two aliquots were combined in a centrifuge tube. Hexane (2 ml) (Rathburn, UK) and water (2 ml) were added and shaken vigorously. The tubes were centrifuged for 10 min at 1000g and 4°C (Heraeus Sepatech, Minifuge, Germany). The organic phase was transferred to 2 ml water, shaken and centrifuged a second time to facilitate the phase separation. The aliquot was dried in a speed-vac (Savant, USA) and dissolved in 1 ml HPLC mobile phase. Prior to injection, samples were filtered through a 0.45 μm PTFE filter to remove lipids and impurities and stored under an atmosphere of nitrogen. All extraction procedures were carried out in subdued light.

The HPLC set-up employed was a Waters (UK) modular system consisting of a U6K injector fitted with a 20μl sample loop, one 501 pump, a 455 spectrophotometer fitted with a Tungsten halogen lamp (Knauer, Germany), a 680 gradient controller and a laptop computer with a Pico data acquisition software (Pico Technology, Cambridge, UK). A CFP 8121 DC pre-amplifier was installed between the spectrophotometer and the data acquisition software. The amplification of this system resulted in the thirty-fold increase of the detector signal. The column was a normal phase 135mm x 4mm LiChrosorb® Si60, 5μm analytical column and a 30mm x 4mm LiChrosorb® Si60, 5μm guard column. Both columns were acidified with a solution of 6-phosphoric acid

(1%) (BDH, UK) in methanol (Rathburn, UK) for one hour. The columns were flushed with mobile phase for 16 hours prior to analysis. Elution was carried out isocratically using 86 hexane: 14 acetone (v:v) as mobile phase at 1.2 $ml.min^{-1}$. All mobile phase solvents were mixed at low pressure and degassed ultrasonically (Townson & Mercer, UK) prior to use. Separation was complete after 30 minutes with no carry-over. All solvents were HPLC grade.

Astaxanthin, canthaxanthin, β-carotene and zeaxanthin were identified with reference to authentic standards (gift of F. Hoffman-La Roche, Switzerland). Lutein was identified with the retention time factor of 1.9 times the astaxanthin retention time."

Pigment profiles of eggs collected in 1996 were analysed by a similar method with the assistance of John Webb (FRS Marine Lab, Aberdeen).

Eggs produced by anadromous female trout could by identified by their high proportion of the marine pigment astaxanthin, relative to eggs of freshwater resident females (Fig. II.1). Identification of eggs of freshwater resident females was based on the presence of lutein and also of relatively high levels of the pigments canthaxanthin and zeaxanthin.

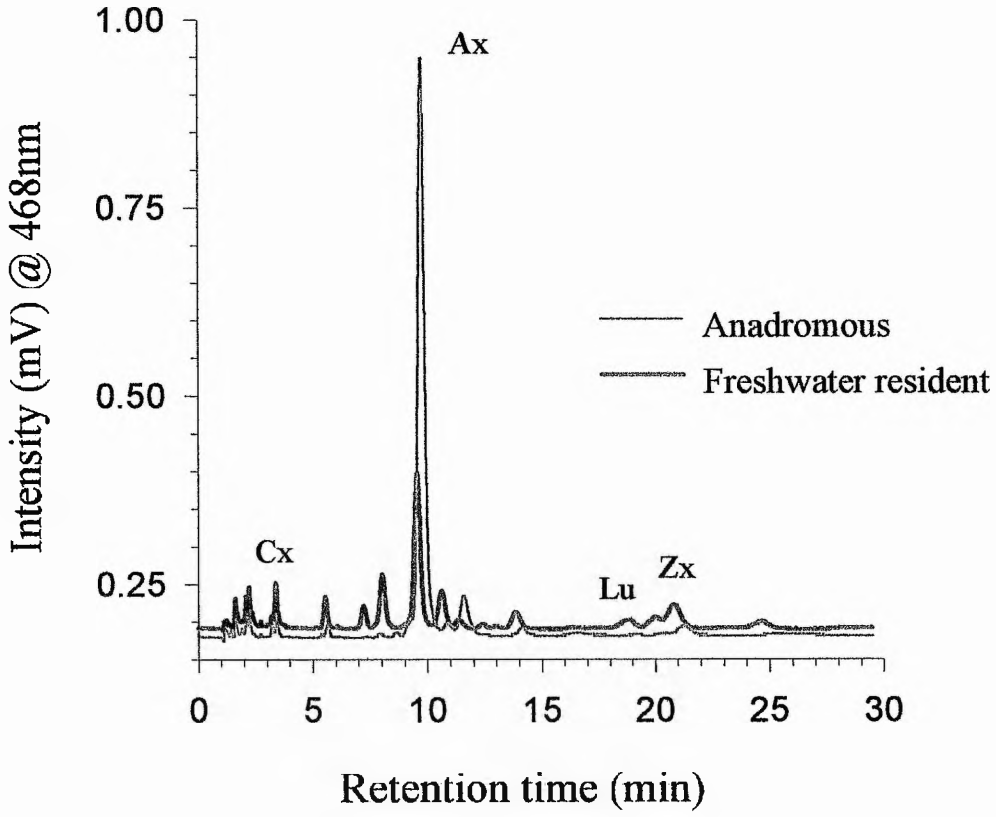



**Fig. II.1.** Typical carotenoid pigment profiles of eggs of anadromous and freshwater resident female trout. Ax: Astaxanthin; Cx: Canthaxanthin; Lu: Lutein; Zx: Zeaxanthin.

## Appendix III: Sample temperature records

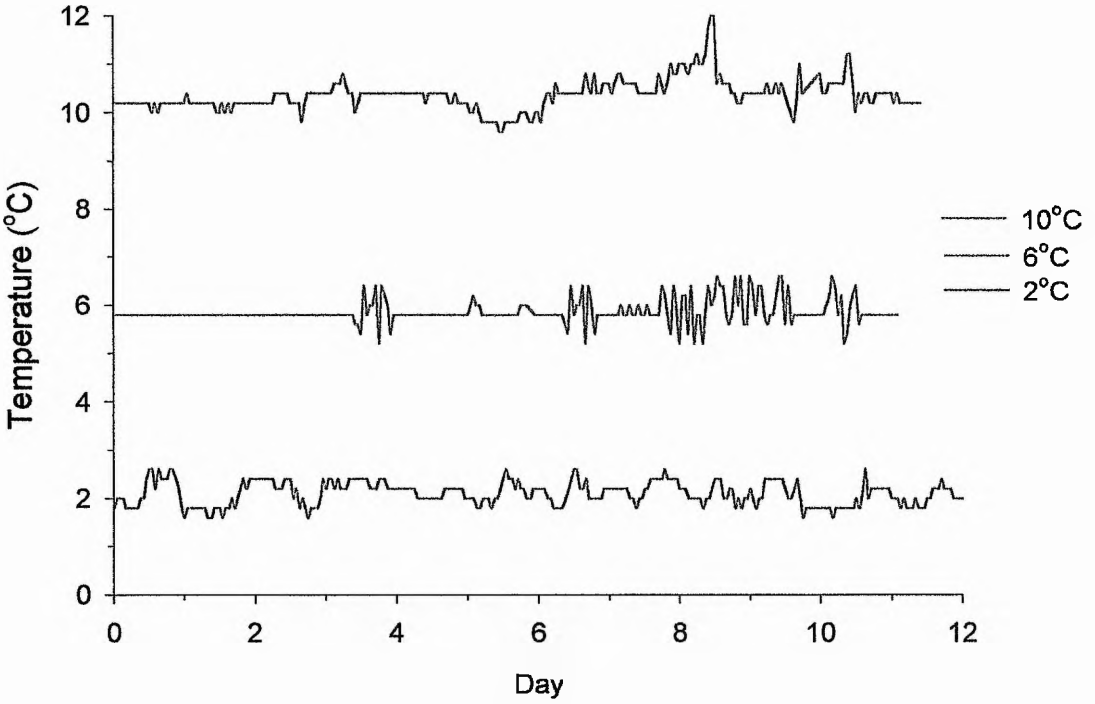



**Fig. III.1.** Sample temperature records from the 10°C, 6°C and 2°C regimes. The 10°C and 6°C records are from the period 04/04/1996 to 15/04/1996; the 2°C records are from the period 07/03/1996 to 19/03/1996. Mean temperatures for these periods were 10.4 ± 0.06°C (95% confidence intervals), 5.88°C ± 0.02°C, and 2.10 ± 0.03°C, respectively.

## Appendix IV: Details of certain frequently-used reagents

| *Buffered 10% formalin* | |
|---|---|
| | (pH 7.2) |
| Formalin | 100ml |
| Sodium dihydrogen phosphate (monohydrated) | 4g |
| Disodium hydrogen phosphate (anhydrous) | 6.5g |
| Distilled water | 900ml |

| *Bouin's fixative* | |
|---|---|
| Picric acid (saturated solution) | 75ml |
| Formalin | 25ml |
| Glacial acetic acid | 5ml |

| *Phosphate-buffered saline (PBS)* | |
|---|---|
| | (pH 7.2) |
| Sodium chloride | 160mM |
| Disodium hydrogen phosphate | 10mM |

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